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ABSTRACT

Antibody Contributes to Heterosubtypic Immunity In the Cotton Rat Model of Influenza

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Influenza virus infection or vaccination evokes an immune response to viral hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, which results in neutralizing antibody against influenza A that is subtype or even strain specific, but not broadly protective. A heterosubtypic immune response that offers protection against a variety of different influenza A subtypes has been induced in experimental animal models, and there are epidemiologic data that suggest a degree of protection may occur in humans. Early studies of this broad cross-protective response indicated that cytotoxic T lymphocytes (CTL) were responsible. However, more recent studies in mice demonstrate that antibodies also contribute to this immune response. We established a model of heterosubtypic immunity (HSI) in cotton rats (*Sigmodon hispidus*) that offer a number of advantages to study influenza pathogenesis and immunity to influenza. Cotton rats were infected with influenza A/PR/8/34 (H1N1) or A/Wuhan/359/95 (H3N2), and then challenged with A/Wuhan/359/95(H3N2) virus 4 weeks later. The results demonstrated measurable heterosubtypic immunity in cotton rats, characterized by enhanced viral

clearance, protection from tachypnea (a reliable measure of lower respiratory infection in this model), a vigorous early cellular recall response, and a reduction in bronchiolar epithelial cell damage. Further studies to elucidate the contribution of antibody to this response showed that cotton rats transfused with H1N1-immune serum prior to challenge with an H3N2 virus were protected from influenza-associated tachypnea, and the quality of protection correlated well with the antibody titer transferred. Immunization with an inactivated preparation of virus delivered intramuscularly also provided protection, suggesting that cell mediated and/or mucosal antibody may not be required for protection in our model. Passive transfer of monoclonal antibody targeting M2e (the extracellular domain of influenza M2 protein), but not nucleoprotein (NP), significantly reduced virus-induced tachypnea suggesting that antibodies specific for conserved epitopes on the virus exterior can mediate this type of protection. A further understanding of methods to induce this type of cross-protective antibody response may lead to the development of more broadly-protective influenza vaccines.

Antibody Contributes to Heterosubtypic Immunity in the Cotton Rat Model of Influenza

by

Timothy M. Straight, MD

A thesis submitted to the Uniformed Services University in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2010

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ANTIBODY CONTRIBUTES TO HETEROSUBTYPIC IMMUNITY IN THE COTTON RAT MODEL OF INFLUENZA

CHAPTER 1

Introduction

Influenza virus epidemics have been devastating to mankind throughout history. Although many documented reports of widespread disease in ancient history were likely due to influenza virus, it is difficult to attribute them to influenza based on the accuracy of the limited historical information available. Most authors agree that a pandemic in 1580 AD was convincingly caused by influenza, and evidence in the historical record includes ten "probable" and three "possible" pandemics since that time (Potter 1998, Potter 2001). Certainly, this virus has consistently been a near-constant threat to public health for a significant portion of recorded history. The most notable pandemic in recent history was the 1918 "Spanish Flu" influenza A pandemic, which caused an estimated 40 to 100 million deaths (Nicholson, Wood, and Zambon 2003; Johnson and Mueller 2002). Following this devastating outbreak, novel viruses emerged and caused pandemics in 1958, 1968, and again in 2009. In addition, there are annual epidemics of this global pathogen with an estimated 20% of all children and 5% of adults worldwide contacting symptomatic influenza A or influenza B each year (Nicholson, Wood, and Zambon 2003). Even in young healthy persons, influenza significantly affects direct healthcare costs, losses in worker productivity, and quality of life (Lee et al. 2002, Demicheli et al. 2001). It is estimated that every year influenza virus infects over 24 million Americans, resulting in 40,000 deaths, and approximately 87 billion dollars in health care costs and lost productivity in the United States (Molinari et al. 2007). Human disease can be

prevented by the prophylactic administration of effective influenza vaccines. However, despite an active vaccination program in the United States, influenza epidemics are an annual occurrence. These outbreaks of disease are particularly evident when viruses emerge that are antigenically distinct from those included in the seasonal vaccine, or from previously circulating viruses. As a consequence, extensive surveillance studies are carried out world-wide in an effort to identify emerging strains that should be included in the vaccine. Influenza vaccines are re-formulated annually, so that strains able to provide protective immunity against current circulating strains can be included as antigens. Despite these vaccine efforts to reduce the annual burden of influenza disease, vaccines developed may mismatch strains that actually circulate the following year, and will offer little to no protection from new viral subtypes. Novel strains may include viruses with a few amino acid changes at important antigenic sites (antigenic drift variants), or viruses that have incorporated hemagglutinin (HA) and/or neuraminidase (NA) genes from a zoonotic source (antigenic shift variant). In 2009, a newly emerged H1N1 virus caused the latest pandemic of influenza disease. This virus had never circulated in humans before, and was antigenically distinct from previously circulating H1N1 viruses. Disease spread quickly, with over 20,000 cases identified by national surveillance efforts in the United States during first two months after the initial outbreak (Centers for Disease Control and Prevention 2009). Although highly infectious, pathogenicity was fortunately less severe than anticipated for a new pandemic strain. Even with less severity, conservative estimates report that from the first case in April through July of 2009, 1.8 million to 5.7 million symptomatic cases of pandemic (H1N1) 2009 occurred in the United States, resulting in 9,000–21,000 hospitalizations, and possibly 800 deaths (Reed

et al. 2009). The Centers for Disease Control and Prevention (CDC) used similar methodology to create estimates at the end of the 2009-2010 season, and reported between 43 million and 89 million cases of 2009 H1N1, 195,000 to 403,000 H1N1-related hospitalizations, and about 8,870 to 18,300 deaths related to pandemic (H1N1) 2009 in the United States (CDC 2010). In addition to the cost of human suffering, there was a significant burden placed on the health care system to accommodate cases or even suspected cases of influenza. Due to antigenic disparity from previously circulating strains, pandemic (H1N1) 2009 virus also evaded detection by rapid diagnostic (enzyme immunoassay) kits commonly used in the hospital setting with test sensitivity reported as low as 17%; leading to difficulty in establishing cases and proper treatment, and increased the use of molecular techniques to identify the virus accurately (Ginocchio et al. 2009, Straight et al. 2010). The seasonal vaccine containing a previously circulating H1N1 strain offered poor protection against this antigenically distinct strain. Recent examination of overall vaccine effectiveness of the trivalent inactivated vaccine (TIV) was found to be between 33 and 55% in military service members studied, while the vaccine effectiveness of the live attenuated influenza vaccine was between 6 and 38% (Johns et al. 2010). Production of a vaccine containing the antigenically-matched H1N1 component was therefore essential to combat cases of “swine flu”. Alternative vaccine strategies that offer protection against a wider range of viruses are needed to more effectively combat the emergence of such novel influenza viruses.

Influenza Background

There are three types of influenza, designated types A, B, and C. However, only types A and B are of significance to man. Influenza A viruses are further categorized into subtypes based on the surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). These variable surface glycoproteins (HA and NA) are the immunodominant antigens against which natural immunity or vaccine-induced antibodies are targeted. Influenza A viruses have 16 known subtypes of HA and 9 known subtypes of NA, but only a few of these subtypes typically circulate in humans (Grebe, Yewdell, and Bennink 2008). Influenza has a segmented genome with 8 gene segments, and 11 possible viral protein products known. These viral proteins include: the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA); transmembrane protein and ion channel membrane protein (M2); and the internal proteins including nucleoprotein (NP), non-structural proteins (NS1), nuclear export protein (NEP, formerly NS2), polymerases (PA, PB1, PB1-F2, PB2), and matrix protein (M1).

Through antigenic drift, new strains of virus evolve by accumulation of point mutations in the surface glycoproteins; this enables these new antigenic variant-strains to evade immune recognition leading to outbreaks during interpandemic years (Nicholson, Wood, and Zambon 2003). The segmented genome of influenza A allows for gene re-assortment producing substantial genetic changes in viral progeny. It is through this process known as “antigenic shift”, that novel surface glycoproteins are produced allowing influenza A virus to completely escape protective immunity and result in pandemic disease.

Influenza B virus also causes infection in man, and has been used in animal models to study host-immune responses. Only one subtype of each surface glycoprotein (HA and NA) are recognized in influenza B (Nicholson, Wood, and Zambon 2003). Protective immunity against different influenza types has not been demonstrated. HA and NA antibodies are usually subtype specific, and the antibodies against NP and M proteins produced after natural infection in humans are type-specific (Couch and Kasel 1983). Studies of cell mediated immune responses to influenza A virus infection in humans also demonstrate type-specificity with several recognized antigens (HA, NA, NP and M protein), and offer no protection from heterologous influenza B virus (Couch and Kasel 1983).

Immunity to Influenza A

Protective immunity to influenza A virus depends on a memory immune response to the surface glycoproteins of the virus, mainly hemagglutinin (HA) and neuraminidase (NA). With regard to protection against re-infection, immunity to the HA molecule plays a pivotal role because antibodies to the globular region of this molecule, when present at sufficient concentration at the site of virus exposure, can neutralize the virus and prevent initiation of infection (Liang et al. 1994). Antibodies directed at neuraminidase (NA) offer protection against disease as well by restricting the spread of the virus within the respiratory tract after infection has been initiated (Kilbourne 2006).

These two surface glycoproteins are the main targets of the trivalent inactivated seasonal influenza vaccine, and the subsequent neutralizing antibodies developed can offer protective immunity. This specific immune response against the HA surface

glycoprotein provides several years of complete protection against homologous virus strains in humans (Couch and Kasel 1983). However, this specific response becomes increasingly obsolete when new viruses are encountered secondary to accumulated point mutations (antigenic drift), and sporadically, by acquisition of an HA gene of a distinct subtype by gene re-assortment (antigenic shift) (Liang et al. 1994).

An early example of the failure of the inactivated influenza vaccine in the setting of a changing virus was demonstrated in epidemiologic data from an influenza outbreak in 1947 at Fort Monmouth, New Jersey. Despite excellent vaccine effectiveness from 1943-1945, the influenza strain used to develop the vaccine was antigenically different from strains circulating among military service members that the resulting antibody response was inadequate to protect a large number of them from influenza disease (Kilbourne 2006).

Another example of the failure of inactivated influenza vaccine to protect against an antigenically different virus was observed during the 2003-2004 influenza season, wherein the live attenuated influenza vaccine (LAIV) appeared to produce a greater degree of protection than the widely-used trivalent inactivated vaccine against drift variant A/Fujian-like H3N2 virus (Mendelman et al. 2004). While the widely-used trivalent inactivated vaccine showed poor protection against this drift variant in culture-proven influenza disease (14% effective among healthcare workers in a small study, and 47% effective in a larger study), the live replicating virus of LAIV was able to produce a much greater degree of cross-protection in the setting of a vaccine mismatch (56% effectiveness within pilot population studied). (Centers for Disease Control and

Prevention, August 2004; Centers for Disease Control and Prevention, January 2004; Halloran et al. 2007)

The two examples discussed above show reduced vaccine effectiveness in the setting of antigenic drift and vaccine mismatch, but protection may be negligible if a completely novel, antigenic shift-variant began to circulate in humans. Protection is predominantly mediated by antibodies against HA, and a sufficient concentration of antibody at the appropriate site can neutralize the virus and prevent infection (Liang et al. 1994). However, in the absence of neutralizing antibodies (e.g. in the setting of a novel influenza strain possessing a new HA glycoprotein), other factors may still contribute to a cross-reactive immune response.

A cross-protective immune response has been well documented in the murine model, but only limited evidence exists that this response is present and active in humans. The inability of current vaccines to protect against antigenic drift and shift variants has led to a quest for a "universal" vaccine. This thesis describes experiments to test the hypothesis that such heterosubtypic immunity (cross-protective immunity across different subtypes) can be induced in the cotton rat model, and to identify immune mechanism(s) that should be exploited in the development of more broadly-protective vaccines.

Demonstration of Heterosubtypic Immunity

Heterosubtypic immunity has been studied for many years beginning in the early 1960's (Schulman and Kilbourne 1965, Henle and Leif 1963). These studies demonstrated the formation of antibodies that were cross-reactive against heterosubtypic influenza viruses, but only after multiple previous infections in animal models.

Schulman and Kilbourne were the first to investigate a cross-reactive immune response irrespective of antibody specificity by evaluating the endpoint of viral titers in the lungs of immunized mice after challenge with a heterosubtypic virus (Schulman and Kilbourne1965). These authors observed that mice previously immunized by a live virus infection developed a partial immunity and protection from influenza disease when challenged with virus of a different subtype. The authors demonstrated that mice infected by exposure to aerosolized influenza A virus were found to be "partially immune" upon challenge with influenza A2 as demonstrated by several outcomes including: less extensive pulmonary lesions, reduced titers of pulmonary virus, and a decrease in mortality when compared to animals previously infected with influenza B virus. This partial immunity was not observed in animals challenged with heterologous influenza B following natural infection with influenza A2. It was also noted that intraperitoneal administration of inactive virus did not induce the same partial immunity despite high titers against homologous virus. Although the investigators did not realize the full implications at the time, these data suggested a cross-reactive component of the host immune response that could not be accounted for by antibodies alone; most likely explained by cellular immune responses, which were just beginning to be understood.

For the next several decades, research focused on the role of the cellular immune response, which was thought to be primarily responsible for this kind of cross-protective immune response. Indeed, CD8+ T cells with specificity for epitopes of conserved proteins do protect mice against heterosubtypic infection (Allan et al. 1990). However, more recent studies in mice have suggested that multiple components of the immune

system contribute to a heterosubtypic immune response, including a more broadly protective antibody response.

In many studies, dramatic reductions in mortality have been observed in previously infected mice challenged with lethal doses of heterosubtypic mouse-adapted strains of influenza A virus (Epstein 1997). Heterosubtypic immunity has been documented in other animal models as well, although with a limited number of studies. Cross-protective immune responses have been demonstrated in ferrets, chickens, and pigs (Yetter, Barber, and Small 1980; Alexander and Parsons 1980; Van Reeth et al. 2003). Epidemiologic data suggests this type of response can also be clinically significant in the setting of human disease. In the subsequent sections, the immune mechanisms that contribute to heterosubtypic immunity are detailed, and a review of studies demonstrating heterosubtypic immunity is provided in Table 1.

Role of the Cell Mediated Response

Early studies of heterosubtypic immunity showed that the cell mediated response plays a role in protection from disease (Yap, Ada, and McKenzie 1978; Lin and Askonas 1980; Lin and Askonas 1981). It has now become clear that the majority of the CD8+ T cell response targets viral antigens that are conserved between subtypes (Liang et al. 1994). Yewdell et al. proposed that a significant portion of cross-reactive T cells in BALB/c mice recognize nucleoprotein (NP), and this has been documented by others as well (Yewdell et al. 1985, Ada and Jones 1986). It was also observed through evaluating T cell precursor frequency that about 30% of influenza-specific T cells indeed recognize NP (Ada and Jones 1986). In has also been demonstrated that the transfer of clonal cytotoxic T lymphocytes targeting a conserved influenza NP antigen can provide

Table 1. Review of studies demonstrating heterosubtypic immunity in animal models

Authors	Model	N/group	Immunization	Route	Challenge	Endpoint(s)	Hetero-subtypic Immunity	Proposed Mechanism
Schulman and Kilbourne 1965	murine	5	A/NWS/33 (H1N1)	Intranasal	A/JAP/305/57 (H2N2)	Viral titers, mortality	(+)	
Yetter, Barber, and Small 1980	ferret	9	A/PR/8/34 (H1N1)	Intranasal	A/PC/73 (H3N2)	Viral titers	(+)	
Alexander and Parsons 1980	chicken	10	A/dk/Alberta/35/76 (H1N1)	Intra-muscular	A/tern/S. Africa/61 (H5N2)	Viral titers, mortality	(+)	
Liang et al. 1994	murine	2-7	A/PR/8/34 (H1N1)	Intranasal	X31(H3N2)*	Viral titers, mortality	(+)	Cell mediated immunity, possible antibody role
Bender et al. 1994	murine	3-5	A/Port Chalmers/1/73 (H3N2)	Intranasal	A/PR/8/34 (H1N1)	Viral titers, CTL activity	(+)	CD8 cells not required, possible antibody role
Epstein et al. 1997	murine	8-9	A/PR/8/34 (H1N1)	Intranasal	X-79 (H3N2)**	Viral titers, mortality	(+)	Possible antibody role, cell mediated immunity not required
Nguyen et al. 1999	murine	10	A/Udorn/307/72 (H3N2)	Intranasal	A/PR/8/34 (H1N1)	CTL activity, weight loss, mortality	(+)	Cell mediated immunity
Benton et al. 2001	murine	10-32	A/PR/8/34 (H1N1)	Intranasal	X-79 (H3N2)**	Viral titers, mortality	(+)	Cell mediated immunity, IgA not required
Nguyen et al. 2001	murine	5	A/Udorn/307/72 (H3N2)	Intranasal	A/PR/8/34 (H1N1)	Viral titers, weight loss, mortality	(+)	Possibly antibody role, cell mediated immunity not required
Van Reeth et al. 2003	porcine	6-10	A/Sw/Flanders/1/98 (H3N2)	Intranasal	A/PR/8/34 (H1N1)	Viral titers	(+)	

*Recombinant virus: A/PR/8/34 X A/Aichi/68 (H3N2)

**Recombinant virus: A/PR/8/34 X A/Philippines/2/82 (H3N2)

heterosubtypic protection from disease in mice (Lukacher, Braciale, and Braciale 1984). Passive transfer of large numbers of *in vitro* activated T cells possessing subtype-specific cytotoxic activity to influenza-infected mice has also been shown to reduce pulmonary virus titers, promote recovery from infection, and provide protection from disease (Nguyen et al. 1999).

Induction of cross-reactive influenza-specific CD8+ T cells by infection or live virus vaccination can improve recovery from subsequent influenza A virus infection (Liang et al. 1994). CD8+ T cell receptors interact with small peptides from the foreign antigen in complex with MHC class I molecules. These complexes form most efficiently when the protein is expressed in the antigen presenting cell. For this reason, peptide/MHC class I complexes are present following infection or live virus vaccination, but are less abundant following vaccination with inactivated (non-replicating) vaccines. The current licensed inactivated vaccines are therefore not likely to induce large numbers of influenza-specific CD8+ T cells.

In addition to data showing that a memory CTL response is sufficient to provide some protection from disease, there are also data that show that other components of adaptive immunity also contribute to heterosubtypic immunity. Depletion of CD8+ T cells with monoclonal antibody reduced, but did not completely remove, protection from disease upon challenge with heterosubtypic virus suggesting another possible mechanism involved in this immune response (Liang et al. 1994). Also, induction of an effective cross-reactive T cell response through immunization has been difficult, and the passive transfer of activated T cells usually requires a tremendous number of cells (e.g. 10^7 cells per mouse) to achieve significant protection against heterosubtypic virus, hardly an

accurate reflection of what may occur in a natural biologic response (Nguyen et al. 1999, and Nguyen et al. 2001). These issues outline the importance of searching for other possible components that may contribute to this cross-protective response.

Role of Circulating Antibody

Studies to clarify the contribution of the CTL to heterosubtypic immunity revealed additional data suggesting a role for antibody, when protection from disease was studied in β_2m (-/-) mice, which lack CD8+, class I MHC-restricted CTLs (Epstein et al. 1997). Eichelberger et al. had previously demonstrated that normal mice depleted of CD8+ T cells via monoclonal antibody and β_2m (-/-) mice were both able to clear virus in the setting of primary influenza A virus infection – clearing virus without functional class I major histocompatibility complex (MHC) glycoproteins and class I MHC restricted, CD8+ effector T cells (Eichelberger et al. 1991). Bender et al. were able to further demonstrate that this held true for a heterosubtypic immune response as well with protection correlating with the development of class II major histocompatibility complex-restricted pulmonary cytotoxic activity, presumably mediated by virus-specific CD4+ T cells (Bender et al. 1994). Epstein et al. also found that a heterosubtypic immune response was still possible in β_2m (-/-) mice, confirming that class I restricted T cells are not required (Epstein et al. 1997). However, these authors additionally noted that depletion of CD4+ T cells only partially abrogated the heterosubtypic immune protection, and that viral replication is partially controlled even in mice with depleted CD4+ and CD8+ T cell populations. This finding that partial cross-protection remained, despite depletion of CD4+ and CD8+ T cell populations, suggested a potential role of cross-protective antibodies.

More recent studies have further indicated that circulating antibodies can indeed have a significant role in heterosubtypic immunity in mice. Antibodies that are cross-reactive for different subtypes acting through the targeting of conserved viral determinants have been suggested by studies in which heterosubtypic immunity could not readily be attributed to T cells (Liang et al. 1994, Epstein et al. 1997). Heterosubtypic immunity is lost in the absence of B cells in some studies in mice, also providing support that heterosubtypic immunity could be mediated by cross-protective antibodies (Nguyen et al. 2001). Nguyen et al. investigated whether groups of mice with either depleted CD8+ T cell population or immunoglobulin μ heavy chain (B cell deficient) would be able to mount a protective heterosubtypic immune response (Nguyen et al. 2001). The CD8+ T cell depleted group developed complete heterosubtypic immunity, and were noted to have cross-reactive antibodies to heterosubtypic virus as well as neutralizing antibodies to the immunizing strain. A protective heterosubtypic immune response was not observed in mice that were B cell deficient even though cross-reactive CTL responses were mounted (Nguyen et al. 2001).

The concept that cross-protective immunity can be mediated by antibody response is also supported from studies of newborn mice. Mbawuike et al. demonstrated that newborn offspring of mice immunized during pregnancy have reduced viral titers in lung tissue and are protected from death following a lethal doses of heterosubtypic influenza A virus at 2 weeks of age (Mbawuike et al. 1990). Other data supporting a role of antibody in heterosubtypic immunity come from passive transfer studies, in which immune-serum generated by live virus immunization confers protection against heterosubtypic virus challenge in mice (Benton et al. 2001).

Studies to further clarify the specificity of cross-protective antibody have been inconsistent. Wraith et al. showed that intraperitoneal or subcutaneous immunization with purified influenza proteins from X-31 (a recombinant sharing internal proteins with PR8, but with surface glycoproteins of the H3N2 subtype) protected mice against a lethal challenge with influenza A/PR8 (H1N1) (Wraithe and Askonas 1985; Wraithe , Vessey, and Askonas 1987). In their initial studies the purified proteins containing an HA/NP fraction was protective from heterosubtypic challenge, but a matrix protein (M) fraction was not protective (Wraithe and Askonas 1985); in their follow-up studies they observed protection from heterosubtypic challenge when purified NP was administered SC. The authors speculated that the protection was entirely mediated by memory T cell response as opposed to antibody, but did not investigate this further. This theory was based on the impression that NP-specific antibodies would be expected to be non-neutralizing, and thus unable to provide any anti-viral activity (Wraithe 1987).

Additional studies by Gerhard et al. to evaluate antibody contribution to protection from influenza virus challenge demonstrated a protective effect of HA antibodies as expected, and reduced viral titers with antibodies to NA and M2; but no significant effect with antibodies to M1 or NP (Gerhard et al. 1997). Although Gerhard et al. studies were against homologous virus challenge, their data still sheds light on whether specific antibodies against conserved proteins can mediate protection from disease.

Epstein et al. found no protection against heterosubtypic challenge from passively transferred antibody against influenza NP in mice (Epstein et al. 1997). Subtype-cross-reactive antibodies were studied in ferrets, and no protection from disease was observed

with recombinant influenza proteins other than HA (Jakeman, Smith, and Sweet 1989). Treaner et al. found that intraperitoneal transfer of monoclonal antibodies against the conserved viral protein M2 (derived from influenza A/WSN/33, H1N1) inhibited viral replication in the lungs of mice upon challenge with heterosubtypic virus (influenza A/Udorn/307/72, H3N2), but not heterologous influenza B (Treanor et al. 1990). Another study demonstrated a reduction in viral titers *in vitro* with antibody to M2, but failed to show any effect on the disease by providing monoclonal M2 antibody to mice infected with heterosubtypic influenza A virus (Palladino et al. 1995). Mozdzanowska et al. studied the activity of M2 antibody in SCID mice and found that antibody against this conserved transmembrane protein resulted in a significant reduction in progeny virus, and inhibited the spread of primary infection – however, the mice were still unable to clear the infection (Mozdzanowska et al. 1999).

In addition to antibodies against the extracellular portion of M2, antibodies with specificity for a conserved region of HA (an area not likely to be involved by antigenic shift or drift) have also been shown to provide relatively broad protective immunity to influenza A challenge (Wei et al. 2010, Sagawa et al. 1996, Steel et al. 2010, Ekiert et al. 2009, Okuno et al. 1993). However, these antibodies to such a conserved epitope are not likely to be produced by natural infection, as the associated antigenic region of HA (the highly conserved “stalk” or “stem”) is generally hidden underneath a large, highly variable, globular “head” that appears to dominate the immune response to infection (Steel 2010). Specifically targeted epitopes of the stem region of HA in these studies likely play a critical role in fusion allowing for virus entry into cells, which explains their conserved amino acid sequence (Ekiert et al. 2009). Studies using a monoclonal antibody

against this HA stem region were able to show neutralization of both H1 and H2 subtypes (Okuno et al. 1993). Eliciting these broadly-reactive antibodies *in vivo*, however, has not been possible in vaccine studies until this year. A new study published in Science demonstrated that a gene-based vaccination in mice followed by a seasonal vaccine (a prime-boost strategy) was able to induce antibodies against such a conserved region of HA, and provided protective immunity across influenza A subtypes (Wei et al. 2010).

Role of Mucosal Antibody

During an experiment using pregnant mice, Mbawuike et al. were able to show that animals receiving a single intraperitoneal injection with a monovalent formalin-inactivated influenza A virus vaccine had offspring protected against a lethal challenge dose of the same influenza A virus subtype, as well as two other subtypes (Mbawuike et al. 1990). Cross-fostering of neonates indicated that protection was conferred by breast milk IgA antibodies (Mbawuike et al. 1990). This finding suggested a role for secretory IgA antibodies in heterosubtypic immunity. The protective role of secretory IgA in local immunity to influenza had already been demonstrated when it was shown that intravenously administered polymeric IgA anti-influenza monoclonal antibody was shown to be selectively transported into nasal secretions and to protect against challenge with the homologous virus; this protection could be also be abrogated by intranasal administration of anti-IgA antiserum (Renegar and Small 1991). Passive antibody studies additionally reported that polymeric IgA is the main, if not the only, isotype protective against homologous influenza challenge in the nose (Benton et al. 2001, Renegar and Small 1991). A role for IgA transport that is dependent on the J chain, which is required for polymeric Ig receptor (pIgR)-mediated transport, had been suggested by results of

studies of virus neutralization in monolayers *in vitro* (Epstein et al. 1997; Mazanec et al. 1992; Mazanec, Coudret, and Fletcher 1995). Possible mechanisms of action for cross-protective mucosal IgA antibodies would include interference with viral replication or assembly during transcytosis mediated by antibodies to conserved internal proteins (Benton et al. 2001). Other studies evaluated the contribution of IgA to the heterosubtypic immune response using J chain knockout mice, and found that these animals remained capable of a heterosubtypic response and protection from disease – showing that polymeric IgR-mediated transport is not required to develop a cross-protective immune response (Epstein et al. 1997). This issue was further studied by Benton et al. in 2001, who found that IgA knockout mice were still able to develop protective heterosubtypic immunity in response to live virus infection. Thus, it appears there is no requirement for mucosal IgA for protection from influenza disease, although the possibility of contribution from mucosal IgA remains (Benton et al. 2001).

Role of Innate Immunity

Although recovery from primary virus infection involves both innate and adaptive responses, memory recall responses play a major role in heterosubtypic immunity as specificity against influenza A virus (versus influenza B) is readily demonstrated (Nguyen et al. 2001). Still some studies have demonstrated partial heterosubtypic immunity in lung tissues despite complete depletion of CD4+ and CD8+ T cells suggesting a possible role for some other effector, such as natural killer (NK) cells, CD4-/CD8- double-negative cells, γδ T cells, or natural killer (NK) T cells (Liang et al. 1994, Benton et al. 2001).

Studies of the contribution of NK cells have reported that depletion of this cell population by anti-asialo-GM1 antibody in mice did not significantly reduce the strength of the heterosubtypic immune response and subsequent protection from disease (Liang et al. 1994). Other authors have examined CD1 knockout mice lacking NK T cells and TCR knockout mice that lack γδ T cells, showing that a heterosubtypic response remains in this setting (Benton et al. 2001). Therefore, NK cells, NK T cells and γδ T cells and are not required for a cross-protective response, but their contribution cannot be completely discounted.

Another possibility is that certain non-specific factors are increased in the setting of heterosubtypic immunity that may enhance tissue repair and recovery from disease (Benton et al. 2001).

Human data

Immunity in man is relatively subtype-specific, but some epidemiologic data suggest there may be some protective heterosubtypic response in humans. Unfortunately, the immune correlates of protection from infection have not been characterized for this. It seems clear that the heterosubtypic immunity in humans is weak or transient, given the susceptibility to infection and illness despite repeated prior infections with other subtypes (Epstein et al. 1997, Steinhoff et al. 1993). Also, human observations lack the high degree of control of infectious challenges of initially naïve hosts, which can be performed in animal models.

Frank et al. studied a cohort of families (557 subjects) who had been followed since the birth of a child to monitor for the incidence of respiratory infections (Frank, Taber, and Wells 1983). These authors were able to report data on subjects who had been

infected sequentially with two circulating strains of a different subtype during the same season. Using serologic data, they determined that this occurred in only about 4-5% of persons studied with the highest rates in school-aged children (Frank, Taber, and Wells 1983). The authors did not observe significant differences between any two illness episodes, based on mean temperature and other mean severity scores. There was not enough data presented in the study to compare incidence rates of particular subtypes between groups of participants who had been previously infected versus those who had not.

A study from Japan reported disease rates among four schools during large sequential influenza epidemics with different viral subtypes (Sonoguchi et al. 1985). Of 91 pupils previously infected with H3N2 virus the previous year, only 59% were noted to have H1N1 infection in the subsequent outbreak. This is compared to a group of 82 pupils not previously infected, wherein 91% were later infected with H1N1virus during the second influenza outbreak (Sonoguchi et al. 1985). Although there is no convincing data, studies like these have introduced the concept that previous infections can perhaps reduce the risk of subsequent symptomatic infection, even by a different strain or subtype of influenza.

More compelling data comes from an epidemiologic study that reported infection rates in participants of the Cleveland Family Study () during an H2N2 pandemic in 1957 resulting from a shift in circulating subtype from H1N1 to H2N2 (Epstein 2006). Only 5.6% (1 of 18) of the adults who had previously had symptomatic influenza A developed influenza from the new H2N2 virus, compared to 55.2% (16 of 29) of the children who had had symptomatic influenza A and contracted it again (Epstein 2006). This suggests

that heterosubtypic immunity may slowly develop over time after multiple exposures to influenza virus.

Another interesting epidemiologic observation is that although several strains or subtypes of influenza may co-circulate, there is usually only one disease peak noted throughout the influenza season (Lavenu, Valleron, and Carrat 2004). Using a mathematical model to explain why this epidemiologic phenomenon occurs, Lavenu et al. concluded that a cross-protection level of 50% (defined as reduction in host risk of infection to one strain after infection by a different strain) would accommodate a single influenza illness peak in temperate countries (Lavenu, Valleron, and Carrat 2004).

Despite the apparent limited induction of heterosubtypic immunity in man from natural infection, or from current vaccine regimens, it would be beneficial to explore and enhance those mechanisms able to elicit such a cross-protective response given the encouraging and overwhelming animal data that suggest it is indeed possible.

Summary of Heterosubtypic Immunity to Influenza and Development of Our Hypothesis

Published observations on the immunologic mechanisms underlying heterosubtypic immunity have been inconsistent, and were obtained using varied experimental systems, mouse strains, and virus preparations, which makes interpretation difficult (Epstein et al. 1997). Furthermore, most of the data on this immune response have come from murine studies, which require the use of animal-adapted strains. This reduces the applicability of this data in discussions of human strains of influenza. Histopathology of the lung tissue in mice is quite variable, making interpretation of

severity of infection difficult (Ottolini et al. 2005). Some viral strains can replicate well without causing disease in the murine lung, while others cause disease with minimal replication (Ottolini et al. 2005). Also, lethal doses of influenza are typically used in murine studies – resulting in mortality as the endpoint, leaving interpretation of data as a simple and qualitative "all or none" response.

Cotton rats (*Sigmodon hispidis*), in contrast to mice, are susceptible to a wide variety of human strains of respiratory viruses, and have been established as a model for the study of influenza pathogenesis (Sadowski et al. 1987, Ottolini et al. 2005). This animal model presents with multiple advantages for the study of immune protection from disease, mainly: 1) cotton rats have reliable histopathology of the lung associated with infection allowing a study of clinical disease severity versus mortality alone, 2) cotton rats are permissive to human strains of influenza virus allowing the study of highly relevant influenza A strains, and 3) tachypnea has been demonstrated as a reliable, measureable response correlated with the degree of epithelial damage in the respiratory tract of cotton rats following infection with influenza (Eichelberger, Prince, and Ottolini 2004). The cotton rat model should provide an excellent model to further study characteristics of host immune response to influenza infection.

Despite extensive studies in various animal models, key mechanisms of the heterosubtypic immune response remain controversial. Traditionally, cross-reactive cell mediated immune response was thought to be primarily responsible for heterosubtypic immunity with multiple studies showing the importance of this type of response providing protection from disease. However, more recent studies indicate that antibodies may also contribute to this broadly active immune response. In fact, several of the

published studies discussed above support the idea of multiple components of the adaptive immune system offering partial protection against a heterosubtypic virus. These mechanisms are likely to complement one another in protecting the host against disease, possibly preventing an excessive cellular response that could be harmful in itself (Liang et al. 1994). A safe and effective universal vaccine is therefore likely to be one that elicits both cell-mediated and antibody-dependent mechanisms.

Given the persistent threat of influenza A virus, and the potential for devastating pandemic from new subtypes, it would be of great benefit to public health to develop vaccine strategies that can optimize such a broadly-protective immune response against new and emerging strains of influenza A. In these studies, we examine the characteristics of a cross-protective immune response in cotton rats, and determine the contribution of antibody to heterosubtypic immunity in this new animal model.

Hypothesis:

Immunization with live influenza A virus induces a humoral response in the cotton rat that provides protection from disease following challenge with heterosubtypic influenza A.

AIM 1

Define endpoints that correlate with heterosubtypic immunity in the cotton rat model of influenza. This specific aim was addressed by comparing virus replication, breathing rates (a reliable physiologic indicator of the severity of lower respiratory infection), and lung pathology after inoculating H1N1-immune and non-immunized cotton rats with an H3N2 virus. Our results are reported in *Evidence of a cross-protective immune response to influenza A in the cotton rat model* by Straight, Ottolini, Prince, and Eichelberger in Vaccine (2006) 24:6264-6271, which is included in this document for reference (Straight et al. 2006).

AIM 2

Identify requirements for induction of heterosubtypic immunity in the cotton rat model with regard to dose and type of immunizing virus. The heterosubtypic immune response in cotton rats was assessed by respiratory rate and histopathology after variation in the dose of immunizing virus, as well as variation in which subtype of influenza A was used as the priming versus challenge virus. Corticosteroids were administered to evaluate whether recruited inflammatory cells were required for this type of protection

from disease. These results are presented in both our published paper (Straight et al. 2006) and in the enclosed discussion section.

AIM 3

Define the contribution of antibody in protecting cotton rats against infection with heterosubtypic influenza A virus. Studies were performed to determine whether antibody was present in immune sera that binds to heterosubtypic virus components, and to further characterize the functional aspects of such a cross-reactive antibody. Passive transfer studies were also completed to determine if this type of protective immunity was transferable *in vivo* to immunologically naive animals. These results are presented in the discussion section as well our published paper *Antibody contributes to heterosubtypic protection against influenza A-induced tachypnea in cotton rats* by Straight, Ottolini, Prince, and Eichelberger in Virology Journal (2008) 5:44, which is included in this document for reference (Straight et al. 2008).

CHAPTER 2

Straight, T.M., M.G. Ottolini, G.A. Prince, and M.C. Eichelberger. 2006. Evidence of a cross-protective immune response to influenza A in the cotton rat model. Vaccine 24: 6264-6271

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Evidence of a cross-protective immune response to influenza A in the cotton rat model☆

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Abstract

Epidemiologic evidence suggests that cross-protective immune responses to influenza A viruses that have different hemagglutinin and neuraminidase subtypes occur in humans. This study characterized this heterosubtypic immunity in cotton rats (*Sigmodon hispidus*). Animals were infected with influenza A/PR/8/34 (H1N1) or A/Wuhan/359/95 (H3N2), and then challenged with A/Wuhan/359/95(H3N2) virus 4 weeks later. Viral titers, respiratory rates, and pathology of the respiratory tract following primary and secondary infection were compared. Cross-protection from heterosubtypic influenza A challenge in cotton rats was characterized by enhanced viral clearance, protection from tachypnea, a vigorous early cellular recall response, and a reduction in bronchiolar epithelial cell damage. Cross-protection was retained in steroid treated animals, in which the inflammatory recall response was minimal. Identification of the mechanisms that contribute to cross-protection in cotton rats may lead to the development of influenza vaccine strategies that are broadly protective.

Keywords: Influenza; Cotton rat; Heterosubtypic immunity

1. Introduction

The threat of pandemic disease highlighted by the recent emergence of H5N1 avian influenza in man, challenges us to search for methods to produce more broadly acting vaccines to influenza A. Cross-protection between subtypes has been demonstrated in animal models [1,2], and epidemiologic evidence suggests that this may also occur in man [3–5]. Exploration of the mechanisms that lead to these broadly reactive responses may enhance efforts toward the development of more effective influenza vaccines.

Heterosubtypic immunity manifests as protection from disease caused by influenza with a particular hemagglutinin (HA) and neuraminidase (NA) subtype (H1N1, for example), following infection or immunization with virus that has different HA and NA subtypes (such as H3N2). A dramatic reduction in mortality has been demonstrated by eliciting heterosubtypic immunity in mice infected with lethal doses of mouse-adapted strains of influenza A. Although cell mediated immune responses that target the conserved antigens of influenza A are generally considered responsible for this more broadly reactive immunity [1,6], there is evidence that the humoral immune response also plays a role [7–9].

Cotton rats (*Sigmodon hispidus*) are unique in their susceptibility to infection with a wide variety of human

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respiratory viruses [10], including human strains of influenza A [11,12]. The severity of respiratory disease in this animal model can be assessed by measuring the frequency of breath by whole body flow plethysmography, as well as by assessing the degree of histopathology in the lungs. The increased respiratory rate (tachypnea) that is observed following viral challenge is prevented by prior exposure to the homologous virus [13]. To determine whether there is evidence of cross-protection in this animal model, we infected cotton rats with a virus that does not have the same HA or NA subtype (heterosubtypic) as the challenge virus. We evaluated respiratory rate, virus replication, and histopathology in the lower respiratory tract of infected animals that were not immune to influenza, or previously immunized with either the homologous or heterosubtypic virus.

2. Materials and methods

2.1. Cotton rats

Male and female inbred *S. hispidus* were obtained from a breeding colony maintained at Virion Systems Inc., Rockville, MD. Animals were seronegative for adventitious viruses. Animals were used at 6–12 weeks of age in protocols that follow federal regulations and were approved by the Institutional Animal Care and Use Committee. Cotton rats in this age-range weigh approximately 100–150 g and were matched by age and weight for use in different groups. Animals were sacrificed by CO_2 asphyxiation for the collection of tissue samples.

2.2. Viruses

Tissue culture-adapted influenza A/PR/8/34 (A/PR8), an H1N1 virus, was obtained from ATCC and grown in a monolayer of MDCK cells resulting in a viral titer of 10^8 $TCID_{50}$/ml. A number of H3N2 influenza A viruses were used in this study, including: X-31 (a reassortant of A/Aichi/68 (H3N2) and A/PR8), A/Memphis/14/98 (A/Memphis), A/California/07/04 (A/California), and A/Wuhan/359/95 (A/Wuhan). The A/Memphis virus was a gift from Dr. Daniel Perez; A/California was obtained from the Centers for Disease Control (Atlanta, GA). All viruses were grown and titrated on MDCK cells. The titer of both A/PR8 and A/Wuhan stock preparations was 10^8 $TCID_{50}$/ml. None of the viruses were passed in cotton rats or in cotton rat cells to adapt them to replicate more efficiently in cotton rats. Virus stocks were stored at −70 °C, and thawed immediately prior to use.

2.3. Viral titration

Viral titrations were performed on monolayers of MDCK cells as previously reported [14]. The titer was recorded as the inverse dilution that resulted in cytopathic effect in 50% of infected wells ($TCID_{50}$) and for lung homogenates is reported per 1 g of tissue. The lowest level of virus detectable in the lung or nasal homogenates was $10^{2.48}$ $TCID_{50}$/g.

2.4. Measurement of respiratory rates

Respiratory rates were measured by unrestrained whole body flow plethysmography (Buxco Electronics Inc., Wilmington, NC) as described previously [13]. After calibration of the 2-chamber apparatus (designed to hold adult rats), one cotton rat was placed in each chamber and airway measurements were continuously recorded over a 5-min period. The mean respiratory rate over the entire 5-min period was calculated. Data from each group was presented as mean breaths per minute (±standard error) for five animals per group as indicated in the text and figures. Percent protection from tachypnea was calculated using mean respiratory rates (RR) from each group; non-immune infected animals represented "no protection" and uninfected animals represented the baseline rate: ([RR of non-immune animals infected − RR of immune animals infected]/[RR of non-immune animals infected − RR of uninfected animals) × 100.

2.5. Evaluation of lung pathology

Lungs were inflated intratracheally with 10% neutral buffered formalin in order to maintain the pulmonary architecture, and stored in this solution for at least 24 h prior to paraffin embedding. Following fixation, 4-μm sections were cut and then stained with hematoxylin and eosin (Histoserv Inc., Rockville, MD). Five parameters of pulmonary pathology were scored in each lung section: peribronchiolitis, alveolitis, interstitial pneumonitis, epithelial damage, and airway debris. Each of these parameters was scored separately by two independent reviewers as previously described [15,16]. The slides were randomized and scored blindly. Scores were based on a scale of severity (0–100%), and were validated by an independent pathologist experienced in respiratory viral pathogenesis.

2.6. Experimental design

Anesthetized animals were immunized by infecting with 1×10^7 $TCID_{50}$ virus per 100 g of animal (intranasal) as previously described [10]. Groups of animals that were not immunized, or immunized with either A/Wuhan/359/95 (H3N2) or A/PR/8/34 (H1N1) were challenged with A/Wuhan/359/95 (H3N2) virus 4 weeks later. Animals treated with steroid as an anti-inflammatory agent, received a 3-day course of 4 mg/kg triamcinolone acetonide (intramuscularly) at the following time-points relative to virus challenge: −24, −4, and +24 h. Respiratory rate in each cotton rat was evaluated by whole body plethysmography. The animals were then sacrificed and viral titers determined in lung and nose tissue and lung pathology evaluated. Unless otherwise stated, five animals were evaluated per group at

every time-point. Lung and nose tissue samples were homogenized in 3 ml of DMEM containing 10% SPG for virus titration, and lung samples for histopathology were stored in 10% formalin.

2.7. Statistical analysis

Mean viral titers were compared using Student's *t*-test, and proportional data with Fisher's exact test. Pathology scores and respiratory rates were assessed for each group by non-parametric Kruskal–Wallis for multiple groups, and Mann–Whitney tests between groups. All analyses were performed using SPSS (Version 11.5 or 12.0) statistical software; *p*-values of <0.05 were considered statistically significant.

3. Results

3.1. Immunization with heterosubtypic virus protects against tachypnea

Groups of animals that were immunized with A/PR/8/34 (H1N1), and a group of non-immune animals, were all challenged with A/Wuhan/359/95 (H3N2) 4 weeks later. Respiratory rates were measured by whole body plethysmography to evaluate the degree of tachypnea following infection on days 1, 2, and 4 post-infection. Five cotton rats were evaluated from each study group per time-point. Peak respiratory rates in infected animals were observed on day 2 and are shown in Fig. 1. Tachypnea was observed in non-immune cotton rats undergoing primary A/Wuhan (H3N2) infection with mean respiratory rates significantly greater than that recorded for uninfected cotton rats ($p < 0.01$). Cotton rats that were previously immunized with A/Wuhan (H3N2) and then challenged with the same virus had a mean respiratory rate significantly less than non-immune A/Wuhan-infected animals ($p < 0.01$). Immunity to the homologous virus therefore provided 88% protection from tachypnea compared to non-immune animals. Cotton rats previously immunized with the heterosubtypic A/PR8 (H1N1) virus and then challenged with A/Wuhan (H3N2) also had significantly lower breathing rates ($p < 0.01$). Heterosubtypic immunity therefore provided 61% protection from tachypnea. These results were reproduced in three separate experiments with significant reduction in tachypnea consistently observed in homologous and heterosubtypic immune animals.

This protection from tachypnea was also observed in animals immunized with A/Wuhan (H3N2) and challenged with A/PR8 (H1N1). While non-immune A/PR8-infected animals had a mean respiratory rate of 511 (±22) breaths/min on day 2 post-infection, animals immunized with A/Wuhan (H3N2) has significantly lower respiratory rates of 407 (±35) breaths/min ($p < 0.04$), providing 44% protection from tachypnea. Similar results were obtained in an experiment that included a number of different H3N2 viruses to immunize cotton rats (Fig. 2). These results demonstrated that cotton rats previously infection with a virus that shares some antigens with the challenge virus (X-31), as well as more recent H3N2 isolates (A/Wuhan, A/Memphis and A/California) had significant protection against increased respiratory rates when infected with the H1N1 A/PR8 virus.

Additional experiments demonstrated that the degree of tachypnea in cotton rats challenged with influenza

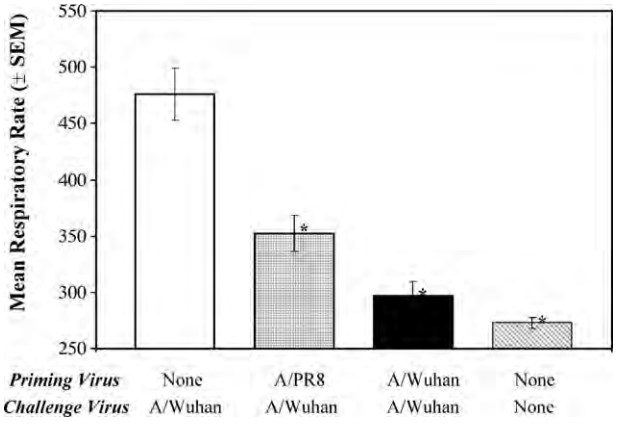

Fig. 1. Mean respiratory rate following influenza A/Wuhan (H3N2) challenge (±S.E.M.). Respiratory rates were measured by whole body plethysmography using five animals per group and 20 uninfected control animals. The mean respiratory rate measured on day 2 after challenge with A/Wuhan (H3N2) is shown for groups that were not either not immune, immunized with heterosubtypic A/PR8 virus, or immunized with homologous A/Wuhan virus. The mean respiratory rate of the uninfected group of animals is also shown. The viruses used to immunize and subsequently challenge the animals are indicated for each group. Groups were compared by Mann–Whitney, and asterisk (*) designates statistical significance when groups are compared to non-immune animals infected with A/Wuhan.

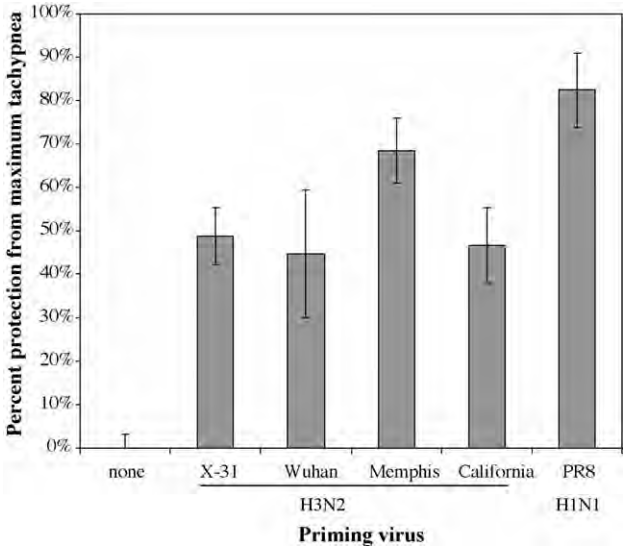

Fig. 2. Percent protection from tachypnea in A/PR8 (H1N1)-challenged cotton rats. Groups of four animals were immunized by intranasal inoculation with approximately 10^6 $TCID_{50}$ A/PR8 (H1N1) or H3N2 viruses, X-31, A/Wuhan, A/Memphis, and A/California. Each of these groups, as well as a non-immune group was challenged 28 days later with 10^7 $TCID_{50}$ A/PR8. Respiratory rates were measured by whole body plethysmography and the percent protection from tachypnea calculated as described in Section 2.

B/HK/73 was not affected by prior immunization with influenza A/Wuhan/359/95. The mean respiratory rate observed following primary infection with B/HK/73 was 392 (±19) breaths/min on day 2 following infection. A similar respiratory rate of 428 (±21) breaths/min was noted in cotton rats infected with B/HK/73 following immunization with A/Wuhan (H3N2).

3.2. Immunization with heterosubtypic virus enhances viral clearance

Groups of animals that were immunized with either A/Wuhan/359/95 (H3N2) or A/PR/8/34 (H1N1), and a group of non-immune animals, were all challenged with A/Wuhan/359/95 (H3N2) virus 4 weeks later. Lung and nose tissues were collected on days 1, 2, 4, and 7 following primary or secondary infection with A/Wuhan (H3N2) or A/PR8 (H1N1) and viral titers determined. Five cotton rats from each group were sacrificed at every time-point.

No virus was detected in the lungs of cotton rats infected with A/Wuhan (H3N2) for the second time, showing complete protection by homologous immunity. All lung samples from non-immune cotton rats with primary A/Wuhan (H3N2) infection contained virus 24 h after infection. While virus was present in lung tissue of animals previously immunized with heterosubtypic A/PR8 (H1N1) virus at 24 h, the titer of virus in these animals was below the limit of detection by 48 h; significantly less ($p < 0.05$) than that of animals without prior immunization (Table 1).

Nasal tissue of all non-immune animals undergoing primary infection with A/Wuhan (H3N2) still contained virus 96 h after infection, whereas cotton rats previously immunized with A/Wuhan (H3N2) had no detectable virus by 24 h. Virus was also detected in all cotton rats immunized with heterosubtypic A/PR8 (H1N1), but the mean viral titers were significantly lower ($p < 0.01$) at 24, 48 and 96 h following infection compared to non-immune rats (Table 1).

The experiment was repeated with more frequent time-points (every 12 h), to allow for greater comparison of viral kinetics between groups. At both 36 and 48 h post-infection, the proportion of A/PR8 (H1N1)-immune cotton rats that contained A/Wuhan (H3N2) in their lungs was less than the proportion of non-immune animals with detectable virus (Table 2). These differences were statistically significant at 48 h ($p < 0.05$), indicating enhanced viral clearance in animals that had been immunized with heterosubtypic virus.

Table 2
Proportion of cotton rats with detectable virus in lung tissue following A/Wuhan (H3N2) challenge

Virus, priming/challenge	Proportion of animals (%) with virus recovered after A/Wuhan challenge			
	24 h	36 h	48 h	96 h
None/Wuhan	100	100	60	0
PR8/Wuhan	100	60	10*	0
Wuhan/Wuhan	0*	0*	0*	0

* The proportion of animals infected was significantly less than that of non-immune infected animals (one-tailed Fisher's exact test, $p < 0.05$).

3.3. Immunization with heterosubtypic virus results in increased inflammatory cell recruitment and protection from epithelial damage

Lung tissue samples from five cotton rats per group were collected on days 1, 2, 4, and 7 post-infection for histopathologic examination. Five categories of pathologic findings were assessed for each sample as described above: peribronchiolitis, alveolitis, interstitial pneumonia, epithelial damage, and airway debris/obstruction. Primary infection with either A/PR8 (H1N1) or A/Wuhan (H3N2) virus led to significant alveolitis, interstitial pneumonia, and epithelial damage. The type and degree of pathology observed was consistent with previous evaluations of lung pathology in cotton rats receiving similar doses of influenza [13,14]. The extent of alveolar and interstitial pathology, as well as airway debris in cotton rats immunized with heterosubtypic A/PR8 (H1N1) did not differ significantly from influenza-infected non-immune animals. Cotton rats immunized with homologous A/Wuhan (H3N2) prior to infection had less severe alveolitis and interstitial pneumonia than rats with primary infection on days 2, 4, and 7 post-infection (results not shown). In contrast, the extent of peribronchiolitis on days 1 and 2 was much greater in homologous-immune animals than that observed following primary infection ($p < 0.01$,

Table 1
Virus titers in cotton rats following A/Wuhan (H3N2) challenge

Virus, priming/challenge	Tissue	Mean virus titer ($\log_{10}$ $TCID_{50}$/g) ± S.E. after A/Wuhan challenge[a]			
		24 h	48 h	96 h	168 h
None/Wuhan	Lung	6.20 ± 0.18	3.09 ± 0.26	<2.48	<2.48
PR8/Wuhan	Lung	6.30 ± 0.18	<2.48*	<2.48	<2.48
Wuhan/Wuhan	Lung	<2.48*	<2.48*	<2.48	<2.48
None/Wuhan	Nose	6.00 ± 0.18	6.75 ± 0.18	5.85 ± 0.18	<2.48
PR8/Wuhan	Nose	4.95 ± 0.09*	4.95 ± 0.09*	5.40 ± 0.20*	<2.48
Wuhan/Wuhan	Nose	<2.48*	<2.48*	<2.48*	<2.48

[a] Virus titers were determined as described in Section 2. The limit of detection was $10^{2.48}$ $TCID_{50}$/g. Each time-point includes data from five animals in each group.

* Data were statistically significant compared to animals that had not previously been immunized ($p < 0.05$, ANOVA and Student's t-test for viral titers).

Table 3
Lung pathology scores from cotton rats following A/Wuhan (H3N2) challenge

Virus, priming/challenge	Category	Mean severity score (%) ± S.E. after A/Wuhan challenge			
		Day 1	Day 2	Day 4	Day 7
None/Wuhan	PB[a]	5 ± 0	25 ± 0	43 ± 18	30 ± 14
PR8/Wuhan	PB	21 ± 4*	70 ± 12*	45 ± 12	26 ± 13
Wuhan/Wuhan	PB	45 ± 12*	85 ± 6*	75 ± 0	20 ± 5
None/Wuhan	ED[b]	35 ± 10	31 ± 12	41 ± 14	13 ± 5
PR8/Wuhan	ED	41 ± 14	37 ± 16	3 ± 1*	13 ± 5[c]
Wuhan/Wuhan	ED	65 ± 10	9 ± 4*	6 ± 5*,[d]	4 ± 1

[a] Peribronchiolitis.
[b] Epithelial damage.
[c] Scores less than 20 reflect very little pathology. In a repeat experiment, the average epithelial damage observed in a similarly treated group of animals was 2 ± 1.
[d] One of five animals in this group showed greater epithelial damage than observed in several repeat experiments and therefore was not included in this average score.
* Data were statistically significant compared to animals that had not been previously immunized ($p < 0.05$, Mann–Whitney).

Table 3), possibly indicating an appropriate recruited memory immune response. This peribronchiolitis peaked by day 2 and decreased over sequential time-points to day 7. A similar early peribronchiolitis was observed in cotton rats immunized with heterosubtypic virus, with a severity score that was significantly greater on days 1 and 2 following challenge compared to that of non-immune cotton rats undergoing primary infection with A/Wuhan ($p < 0.02$). Peribronchiolitis in the heterosubtypic-immune animals also peaked on day 2 following challenge. Peribronchiolitis after primary infection was less severe, and peaked at a later time-point (day 4) following challenge. Photomicrographs of normal cotton rat lung tissue and severe peribronchiolitis are displayed in Fig. 3 as an example of differences that were observed.

Bronchiolar epithelial damage subsequent to primary infection was present to a moderate degree during most of the course of infection (Table 3). Epithelial damage was greatest in animals with homologous immunity on day 1 following challenge, but the scores were not statistically different from that observed in primary infection and rapidly subsided by day 2. Substantial epithelial damage was retained in non-immune animals until day 4 post-infection, whereas significantly less damage was observed in heterosubtypic-immune animals. These differences were statistically significant ($p < 0.02$). Examples of the bronchiolar epithelium with and without damage are displayed in Fig. 4.

3.4. Heterosubtypic immunity is retained in the absence of an inflammatory response

The inflammatory response following influenza virus infection is dramatically reduced by treatment of cotton rats with corticosteroids [14]. Such treatment does not significantly diminish influenza-induced tachypnea [13], demonstrating that the inflammatory response itself does not contribute to changes in respiratory rates. To determine whether

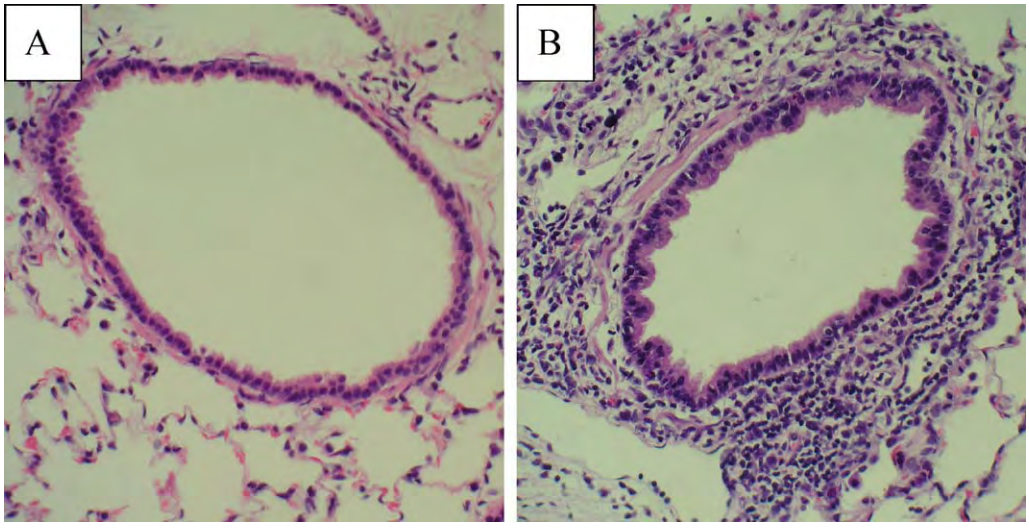

Fig. 3. Representative lung histopathology (200×) demonstrating: (A) severity score of 0%: a normal cotton rat lung bronchiole and surrounding alveoli from an uninfected specimen, and (B) severity score of 100% with robust peribronchiolitis observed in an A/Wuhan-infected animal that had previously been immunized with homologous virus.

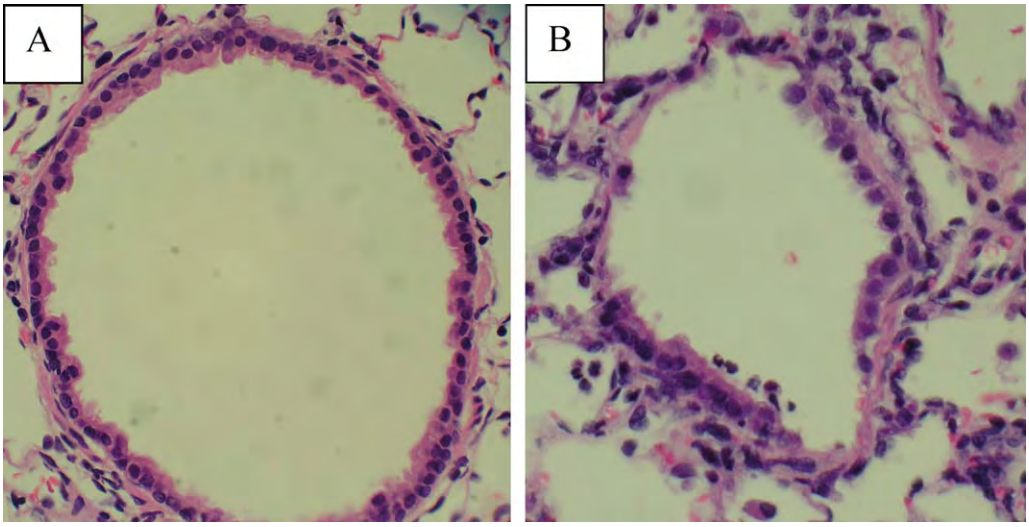

Fig. 4. Representative lung histopathology (200×) demonstrating: (A) severity score of 0% with a normal bronchiolar epithelial cell lining as seen in uninfected animals, and (B) severity score of 100% with severe epithelial damage following A/Wuhan challenge in an animal that was not immune.

heterosubtypic immunity requires recruitment of cells to the lung, we administered non-immune and immune animals with triamcinolone acetonide and challenged them with A/PR8 (H1N1). Five cotton rats were evaluated from each study group. Tachypnea in heterosubtypic-immune animals was less than that recorded in animals exposed to A/PR8 for the first time with respiratory rates of 390 (±21) and 471 (±17) breaths/min, respectively ($p < 0.01$, Fig. 5). The degree of tachypnea observed in non-immune animals was similar to that recorded in non-immune animals without steroid administration in the same experiment (results not shown). While respiratory rate was not significantly affected by the presence of steroid in animals previously immunized, the degree of peribronchiolitis was dramatically reduced with a mean severity score of $13 \pm 5\%$ versus $55 \pm 12\%$ in the absence of steroid ($p < 0.02$). The degree of peribronchiolitis in heterosubtypic-immune animals with steroid administration was similar to that observed in non-immune animals with primary infection-demonstrating that the prophylactic administration of steroids suppressed the inflammation associated with the memory immune response. Similar to respiratory rate measurements, epithelial damage was not significantly affected by the presence of steroid (results not shown).

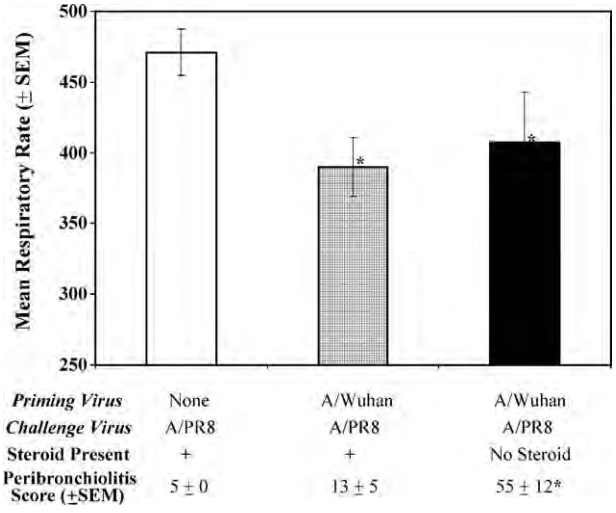

Fig. 5. Mean respiratory rate and mean peribronchiolitis severity scores following challenge of naïve and A/Wuhan (H3N2)-immune cotton rats that had been either treated with triamcinolone acetonide (4 mg/kg/day) or left untreated. There were five animals per group at each time-point. Respiratory rates were measured by whole body plethysmography on day 2 post-infection, and pathology of formalin-fixed lung sections was scored for degree of peribronchiolitis on day 1 post-infection. Groups were compared by Mann–Whitney, and asterisk (*) designates statistical significance when groups are compared to non-immune animals challenged with A/PR8.

4. Discussion

Heterosubtypic immunity provides protection from disease caused by different subtypes of influenza A. It has been suggested that this may explain the existence of a single peak of human illness, during seasons in which different subtypes are known to be circulating and causing disease [17]. A report of cross-subtype protection in humans during either concurrent or successive H3N2 and H1N1 epidemics in Japanese high schools revealed that a significantly smaller proportion of students were infected with H1N1 following an H3N2 epidemic compared to students without previous exposure to the H3N2 virus [4]. Further evidence suggesting a heterosubtypic immune response exists in humans was obtained after analysis of archived sera and review of detailed clinical records collected during the 1957 influenza A pandemic, which showed that adults with previous exposure to H1N1 viruses were less susceptible to the new circulating H2N2 virus [5]. Vaccines able to exploit such an immune response may be useful assets against two of the greatest challenges in successful influenza vaccine development—the antigenic

shift and drift of influenza virus. A further understanding of the mechanisms that contribute to cross-protective immunity may lead toward methods to elicit a more broadly acting immune response to influenza.

In this study, we have demonstrated heterosubtypic immunity to influenza A in the cotton rat model. This is a general finding, with protection from tachypnea observed after infection with a broad range of influenza A viruses. The characteristics of heterosubtypic immunity were explored in detail using A/Wuhan-immune cotton rats that were challenged with A/PR8. The kinetics of virus replication, histopathology and temperature changes after primary infection with A/Wuhan have previously been described and demonstrate a semi-permissive model of infection [11]. As in our prior experiments, we use a high dose of virus (10^7 $TCID_{50}$/100 g animal) to infect animals in both primary and secondary inoculations. However, infection with A/Wuhan at 10^4 $TCID_{50}$/100 g is equally able to provide heterosubtypic immunity against A/PR8.

Use of the cotton rat to study heterosubtypic immunity has several advantages. First, *S. hispidus* is semi-permissive to unadapted influenza strains that infect humans, allowing a model to evaluate cross-protection against relevant challenge viruses. Second, cotton rats develop severe pathology and systemic symptoms of illness in response to these pathogens, which provides practical endpoints to evaluate the degree of protection from influenza. Physiologic measures of respiratory response and histopathologic endpoints add depth to the assessment of disease protection, as opposed to the "all or none" approach of assessing mortality after a lethal dose. These outcome parameters may reveal insight into the mechanisms that result in heterosubtypic immunity.

Our studies show that heterosubtypic immunity in the cotton rat is characterized by protection from tachypnea, enhanced clearance of virus, robust peribronchiolitis, and decreased epithelial damage. These data provide evidence of both an immediate and specific memory response. We will use these endpoints to examine the contribution of different immune mechanisms to heterosubtypic immunity.

Despite several published studies of heterosubtypic immunity in other animal models (murine [1,2,6,18], porcine [19], and ferret [20]), factors implicated in this immune response are inconsistent, perhaps due to different experimental conditions and end-points. Much of the current knowledge about immune effectors involved in heterosubtypic immunity is based on observed responses to infection following deletion of various components of the natural host immune response. While heterosubtypic immunity can be provided by cytotoxic T cells (CTL) that target the conserved internal proteins of the virus, studies clearly show that antibodies can also contribute to cross-protection [1,7,8]. In our studies, the use of corticosteroids to diminish the recruited inflammatory response support the idea that heterosubtypic immunity in cotton rats is mediated by a cellular component that resides at the effector site and/or by antibodies that are not influenced by the use of steroid.

Antibodies with specificity for a conserved peptide of the viral M2 proton channel are an example of those that may offer cross-protection. Studies to evaluate protection from influenza A in mice following passive transfer of monoclonal antibody [21] or immunization with recombinant M2 or M2 peptide conjugate vaccines [22–24] suggest that this is a suitable strategy to induce protective heterosubtypic responses. However, it is unclear what role anti-M2 antibodies play in heterosubtypic immunity following natural infection. We plan further studies to evaluate the role of anti-M2 antibodies and other mechanisms in heterosubtypic immunity in the cotton rat.

This is the first report that demonstrates heterosubtypic immunity to influenza in cotton rats. We have demonstrated that this is characterized by enhanced viral clearance, protection from tachypnea, a vigorous early cellular recall response, and a reduction in bronchiolar epithelial cell damage. This model therefore provides a useful tool to evaluate novel vaccines that induce broadly reactive immune responses to combat the persistent threat of emerging strains of influenza A.

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CHAPTER 3

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Research

Antibody contributes to heterosubtypic protection against influenza A-induced tachypnea in cotton rats

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Abstract

Background: Influenza virus infection or vaccination evokes an antibody response to viral hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, which results in immunity against influenza A viruses of the same HA and NA subtype. A heterosubtypic immune response that offers some protection against different influenza A subtypes has been suggested from epidemiologic studies in human influenza outbreaks, and has been induced in experimental animal models. Original studies of such cross-protection showed that cytotoxic T lymphocytes (CTL) protect H3N2-immune mice from a lethal H1N1 infection. More recent studies in mice demonstrate that antibodies also contribute to heterosubtypic immunity (HSI). We previously demonstrated that HSI in cotton rats (*Sigmodon hispidus*) is characterized by protection of H3N2-immune animals from influenza H1N1-induced increase in respiratory rate (tachypnea). Alternatively, H1N1-immune animals are protected from H3N2-induced tachypnea. The experiments described in this report were designed to elucidate the immune mechanism that prevents this very early sign of disease.

Results: Our results show that cotton rats provided with H1N1-immune serum prior to challenge with an H3N2 virus were protected from influenza-associated tachypnea, with the degree of protection correlating with the antibody titer transferred. Immunization with an inactivated preparation of virus delivered intramuscularly also provided some protection suggesting that CTL and/or mucosal antibody responses are not required for protection. Antibodies specific for conserved epitopes present on the virus exterior are likely to facilitate this protection since prophylactic treatment of cotton rats with anti-M2e (the extracellular domain of M2) but not anti-nucleoprotein (NP) reduced virus-induced tachypnea.

Conclusion: In the cotton rat model of heterosubtypic immunity, humoral immunity plays a role in protecting animals from influenza-induced tachypea. Partial protection against respiratory disease caused by different influenza A subtypes can be attained with either live virus administered intranasally or inactivated virus delivered intramuscularly suggesting that either vaccine regimen may provide some protection against potential pandemic outbreaks in humans.

Background

Influenza A remains a major burden on mankind with annual epidemics of disease and continued potential for devastating pandemics such as that seen in 1918. Neutralizing antibodies that are specific for viral hemagglutinin (HA) and neuraminidase (NA) are induced following immunization with inactivated influenza vaccines and correlate with protective immunity against influenza strains of the same subtype. These specific antibodies do not offer protection against viruses that have a different HA and NA subtype, as noted in the vaccine failure in 1947 when an H1N1 virus emerged that was serologically distinct from the 1943 H1N1 strain used in the vaccine [1]. A more recent example of limited reactivity with a drifted influenza strain occurred in the 2003–2004 season when the vaccine contained an H3N2 virus that was antigenically distinct from newly circulating A/Fujian strain [2]. During this particular season it appeared that the live attenuated vaccine provided individuals with some protection against drifted strains of influenza [3], suggesting that a replicating virus administered intranasally is more likely to induce more broadly acting antibodies or cross-reactive cellular immune mechanisms that can act at the site of infection.

While immunity to influenza is primarily type and subtype-specific, epidemiologic evidence suggests that heterosubtypic immunity can be induced in man [4]. Retrospective studies that show a lower incidence of H2N2 influenza disease in persons previously infected with an H1N1 virus also support this idea [5]. However, the immune responses that correlate with protection of humans against infection with an influenza virus that is of a different subtype have not been characterized. Studies in influenza-infected mice suggest that multiple mechanisms may contribute to this type of protection. Traditionally, cell mediated immune mechanisms against conserved antigen targets have been considered responsible for a cross-protective immune response [6,7]. In contrast, more recent studies demonstrate a role for antibody in heterosubtypic immunity in mice [8,9]. These studies suggest that the magnitude of the immune response as well as the route of immunization is important in establishing antibody-mediated cross-protection.

The specificity of antibodies that provide protection against different influenza A subtypes are likely to be non-neutralizing, since antibodies that block HA-binding or inhibit NA activity are generally thought of as subtype-specific. These could include antibodies that recognize conserved portions of surface glycoproteins or antigens in the viral core. Examples of potential epitopes include a conserved peptide at the cleavage site of the influenza B HA molecule (this peptide has been used to induce immunity against influenza B strains that are antigenically distinct [10]) and the conserved extracellular peptide of M2 (M2e). It has been demonstrated that a monoclonal antibody with specificity for M2e inhibits influenza replication in mice [11] and that a M2e vaccine protects against lethal challenge with both H1N1 and H3N2 influenza A viruses in mice, and reduces shedding of viruses in ferrets [12].

We have used the cotton rat (*Sigmodon hispidus*) to study influenza pathogenesis and immunity. This unique model has the distinct advantage of exhibiting increased respiratory rate (tachypnea) following infection with influenza, a response that is dependent on virus dose and immune status. Respiratory rates are easily monitored by whole body plethysmography, making this a practical end-point to evaluate protection from influenza-induced respiratory disease or vaccine efficacy. We previously established that cotton rats can be used as a model to study heterosubtypic immunity against influenza A; animals exposed to one subtype of virus are protected from respiratory disease upon exposure to a different subtype of influenza A [13]. This protection is retained when animals are treated with steroid to inhibit the inflammatory response, suggesting that heterosubtypic immunity is not dependent on a recruited cellular response. In this report, we show that protection against influenza-induced tachypnea is transferred in serum from animals previously infected with an influenza virus of a different subtype, and examine the potential specificity of the cross-protective antibodies, as well as the route of immunization required to induce heterosubtypic immunity.

Results

Cross-protection is observed following the prophylactic transfer of serum from immunized animals to naïve cotton rats

Previous studies in our laboratory demonstrated that protection from respiratory disease was retained in immune animals after the administration of systemic steroids, which inhibited the acute inflammatory response following challenge with a heterosubtypic virus [14]. These results suggested that the heterosubtypic immune response was not mediated by recruited cells, but rather by local cells at the site of infection or cross-reactive antibodies. To further evaluate whether antibodies play a role in heterosubtypic immunity, we transferred serum from H1N1 or H3N2-immune cotton rats into naïve cotton rats 24 hr before intra-nasal (i.n.) challenge with 10^7 $TCID_{50}$/100 g A/Wuhan/95, an H3N2 virus. Respiratory rates (RR) were measured 1 and 2 days later by whole body plethysmography.

The group of animals that received H3N2-immune serum prior to viral challenge with H3N2 virus was significantly protected ($p < 0.03$) from the effects of respiratory disease

compared to the group undergoing primary infection. The challenge group that was previously infected with the homotypic H3N2 virus was also protected from virus-induced tachypnea ($p < 0.02$). Passive transfer of H1N1-immune serum into 4 animals resulted in a strong trend toward protection, but the respiratory rates measured were not significantly different from the those measured in non-immune animals ($p = 0.06$). These results are presented in Fig. 1 as the mean percent protection from H3N2-induced tachypnea, with respiratory rates for day 2 post-challenge provided in the figure legend.

Variation in the degree of protection in recipients of H1N1-immune serum suggested that the i.p. inoculation of serum may not always transfer an equal amount of antibody into the circulation. To assess the quantity of antibody transferred in each animal, we measured hemagglutination inhibition (HAI) titers in the serum of recipients 12 hr after intraperitoneal (i.p.) transfer of immune sera. The degree of protection from tachypnea correlated with the recipient's pre-challenge HAI titer (Fig. 2A), with Spearman's correlation coefficient of -0.71 ($p < 0.02$). In general, animals with higher HAI titers demonstrated lower RR than recipients of naïve serum. In subsequent passive transfer studies, only animals with an HAI

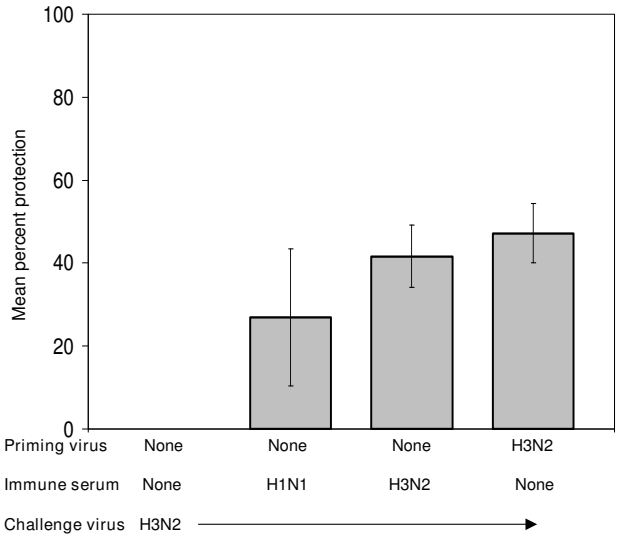

Figure 1
Transfer of H1N1-immune serum protects recipient cotton rats against H3N2-induced tachypnea. Mean percent protection (± SEM) is shown for animals that received H1N1 or H3N2-immune sera and were then challenged with an H3N2 virus, A/Wuhan/95. The immune sera were obtained from cotton rats previously infected with A/PR/8/34 (H1N1) or A/Wuhan/95 (H3N2). Peak respiratory rates were measured on day 2 after challenge and were used to calculate the mean percent protection from virus-induced tachypnea shown in the figure.

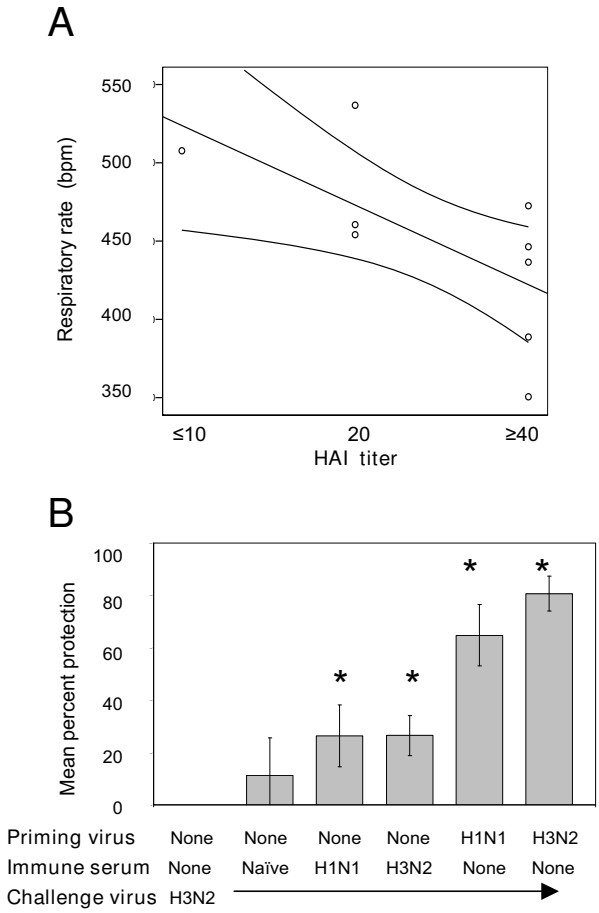

Figure 2
Correlation of protection against tachypnea and HAI titer after passive transfer of heterosubtypic immune sera. Respiratory rates (breaths per minute) and serum HAI titers are shown for individual animals in A. These animals were challenged with A/Wuhan/95 (H3N2) after receipt of H1N1-immune sera. The best fit line and 95% confidence intervals are displayed in the figure. The Spearman's correlation coefficient was -0.71 ($p < 0.02$). Percent protection from tachypnea for groups of animals that received immune sera before H3N2 challenge is shown in B. These groups included animals that did not receive serum, or groups that received from naïve, H1N1-immune or H3N2-immune animals. The mean protection was calculated using results from animals that had HAI titer ≥ 40 following serum transfer. Results are also shown for control groups that were immune to the homotypic or heterosubtypic virus at the time of challenge. Percent protection of different groups were compared by Mann-Whitney test, with statistical significant differences ($p < 0.05$) with the group experiencing primary infection in the absence of immune serum marked with a *.

titer of 40 or greater were considered successful transfer recipients and an HAI titer ≥ 40 was a prerequisite for including individual animal results in the data analysis.

Data collected 2 days post-infection in one such experiment are displayed in Fig. 2B, showing mean percent protection calculated from the mean respiratory rates provided for each animal group in the figure legend. Statistical analysis showed that the RR of animals receiving either heterosubtypic (A/PR/8/34)-immune or homologous (A/Wuhan/95)-immune-serum were significantly less than naïve animals undergoing primary infection ($p < 0.03$ and $p < 0.01$ respectively). Previous studies show that tachypnea is close to resolution by day 4 post-infection and therefore respiratory rates were not measured at this time point. At this late time point, animals did not exhibit any gross difficulty in breathing, and did not have increased histopathology, suggesting that there was no exacerbation of disease. Animals administered non-immune serum prior to transfer did not differ significantly from animals undergoing primary disease ($p = 0.24$).

Neutralizing antibodies in serum of immune cotton rats are subtype specific

To evaluate whether antibodies with hemagglutination inhibition activity contribute to this *in vivo* cross-protection, we examined the ability of serum from H1N1-immune animals (the same pool of serum that had been used in the transfer study) to inhibit agglutination of red blood cells by A/Wuhan/95 (H3N2). The pooled serum had an HAI titer of 640 against A/PR/8/34 but <10 against A/Wuhan/95 (Table 1). This lack of cross-reactivity is expected, indicative of a subtype-specific neutralizing antibody response. To evaluate whether the antibodies that neutralize virus replication are truly subtype-specific in this model, we also determined the amount of antibody required to inhibit replication of H1N1 or H3N2 viruses in MDCK cells. The tissue-culture neutralizing titer for H1N1-immune serum in this assay was 1600 against A/PR/8/34 and <100 against A/Wuhan/95. Because complement component C1q can enhance the activity of antibodies [15], the neutralization assay was also performed in the presence of complement. Addition of C1q increased the neutralizing antibody titer to 3200 but did not change the specificity of the inhibition. A pool of serum from A/Wuhan/95-immune animals showed similar subtype specificity, with a titer of 200 against A/Wuhan/95 that increased to 800 in the presence of complement. Even in the presence of complement, this serum did not inhibit A/PR/8/34 replication at the lowest dilution of antibody used (1/100). Antibodies that inhibited NA activity were also subtype specific; the NA inhibition (NI) titer of H1N1-immune serum that had been used in transfer studies was 80 against A/PR/8/34 and no detectable inhibition was measured against the N2 activity of A/Wuhan/95. The NI titer of H3N2-immune serum was 320 against A/Wuhan/95 and there was no detectable inhibition against the NI activity of A/PR/8/34.

Protection from virus-induced tachypnea is achieved by prophylactic administration of antibodies specific for viral M2 but not viral NP

Antibody with specificity for M2e provides protection against influenza A replication in mice, and therefore has the potential to play a role in reducing tachypnea following infection of cotton rats. To test whether this is the case, groups of cotton rats were treated (i.p. inoculation) with 100 μg monoclonal antibody specific for either influenza nucleoprotein (NP) or M2e 6 hr before infection with A/Wuhan/95 (10^7 $TCID_{50}$/100 g). Four animals were used in each group. Cotton rats that received anti-M2e, but not anti-NP prior to challenge were subsequently protected from tachypnea an ($p < 0.04$, and $p < 0.48$, respectively). These results are shown in Fig. 3.

Heterosubtypic immunity is observed following immunization with UV-inactivated virus that is delivered intramuscularly, and does not require immunization with live virus

Since our cotton rat model of heterosubtypic immunity was established using live virus to vaccinate cotton rats i.n., we examined the ability of inactivated virus to protect animals from virus-induced tachypnea. We also determined whether mucosal immunization was essential to

Table 1: Subtype-specific antibody responses are evident in sera from A/PR/8/34(H1N1) and A/Wuhan/95(H3N2)-infected animals.

	Antibody titer as measured by[a]							
	HAI		*NI*		*Neutralization*		*Neut + C1q*	
Serum source	*H1N1*	*H3N2*	*H1N1*	*H3N2*	*H1N1*	*H3N2*	*H1N1*	*H3N2*
Naïve serum	<10	<10	0	0	<100	<100	<100	<100
H1N1-immune	640	<10	80	0	1600	<100	3200	<100
H3N2-immune	<10	160	0	320	<100	200	<100	800

[a]Standard hemagglutination inhibition (HAI), neuraminidase inhibition (NI) and neutralization (neut) assays in the absence as well as presence of complement factor C1q were performed as described in Materials and Methods. Viruses used for these assays were A/PR/8/34 (H1N1) and A/Wuhan/359/95 (H3N2) that had been used to infect the cotton rats that were the source of this serum pool. Animals were boosted several times by rechallenging them with the same virus before serum was collected. The lowest dilution of serum used in the HAI assay was 1/10 and therefore no inhibition of agglutination is recorded as a titer of < 10. The lowest dilution of serum used in the neutralization assay was 1/100 and therefore no neutralization is recorded as a titer of < 100.

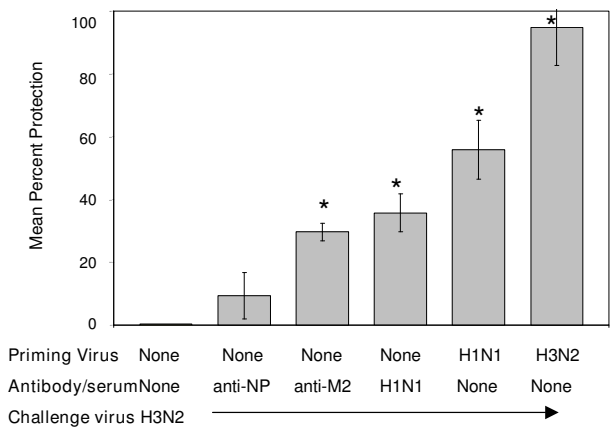

Figure 3
Antibodies specific for M2 but not NP protect against influenza-induced tachypnea. Groups of 6 animals were inoculated i.p. with 100 μg monoclonal antibody (anti-M2 or anti-NP) prepared in saline solution 24 hr before infection with A/Wuhan/95 (H3N2). Control groups of animals underwent passive transfer of 0.5 ml (i.p.) of serum from H1N1-immune animals, or were either infected with the same H3N2 virus or A/PR/8/34 (H1N1) virus 28 days earlier. The percent protection was calculated from RR measured by whole body plethysmography. Groups of animals that had RR statistically different ($p < 0.05$) from animals undergoing primary influenza infection are designated in the figure with an *.

induce heterosubtypic immunity by comparing protection in animals that have been vaccinated i.n. and intramuscularly (i.m.). Since protection against tachypnea was successfully transferred in serum from animals that were immune to heterosubtypic virus, we expected that transudated rather than local mucosal antibodies were responsible for this protection. The A/PR/8/34 virus was inactivated by exposure to UV-light and its inability to replicate verified by titration in MDCK cells. Equivalent amounts of virus (10^7 $TCID_{50}$/100 g) were used to inoculate groups of animals (4 animals per group) i.n. and i.m. with live or inactivated virus. Serum samples were obtained from all animals 2 weeks after immunization to evaluate immune responses by measuring HAI titers.

As expected, exposure to live virus administered i.n. resulted in greater HAI titers than exposure to inactivated virus. Groups of cotton rats that were immunized with the inactivated H1N1 virus were therefore boosted 3 times with this virus preparation at 3 week intervals. At the time of intranasal virus challenge with the heterosubtypic A/Wuhan/95 virus, there was no inhibition of A/Wuhan/95 agglutination of chicken red blood cells. The serum HAI geometric mean titers (GMT) against A/PR/8/34 varied substantially in each of the groups (4 animals per group): 11 following i.n. immunization with inactivated virus; 28 following i.m. immunization with inactivated virus; 100 following i.n. inoculation with live virus; 82 following i.m. inoculation with live virus. The HAI titer in sera of cotton rats infected once with A/Wuhan/95 that served as a homotypic control group, was 57. As expected, this serum did not inhibit agglutination with the H1N1 virus. Protection from influenza-induced tachypnea was observed in the groups of animals immunized i.m. with either live or inactivated virus preparations (Fig. 4), indicating that a local immune response was not required to provide cross-protection. Protection against tachypnea was not observed in the group of animals immunized intranasally with inactivated virus. This group had the lowest HAI titer, suggesting that insufficient titers of cross-protective antibodies had been attained under these conditions.

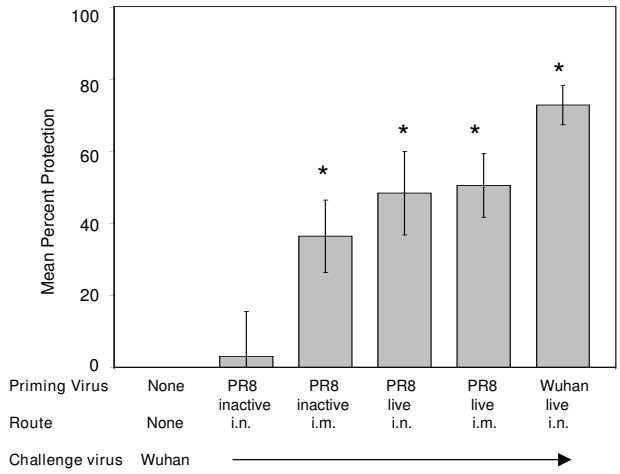

Figure 4
Intramuscular immunization with inactivated H1N1 virus protects against H3N2-induced tachypnea. Groups of animals (4 cotton rats per group) were inoculated with the equivalent of 10^7 $TCID_{50}$ A/PR/8/34 (H1N1) per 100 g. Both live and UV-inactivated virus preparations were inoculated intranasally (i.n.) or intramuscularly (i.m.). Animals in groups immunized with inactivated virus were boosted at week 3 and 6. HAI titers of serum samples obtained by retro-orbital bleed 2 weeks following the final immunization are included in the text. All groups were challenged 10 weeks following the first immunization with A/Wuhan/95 (H3N2). Control groups included naïve animals that provided baseline RR, naïve animals infected with A/Wuhan/95 for the first time, and A/Wuhan/95-challenged H3N2-immune cotton rats. RR were measured by whole body plethysmography and the percent protection from tachypnea calculated for each animal. Protection that was statistically greater than non-immune animals ($p < 0.05$) is marked with an *.

Discussion

Heterosubtypic immunity in man has been suggested from epidemiologic studies of human outbreaks of influenza A [4,5,16]. Identification of the immune components necessary for a heterosubtypic immune response will be critical in the development of more broadly protective vaccines effective against influenza A virus. Both antibodies and cytotoxic T cells have been implicated in cross-protective immune responses in murine models of influenza infection, where the most often used end-point is mortality.

In the cotton rat model, we previously demonstrated that respiratory rate can be used as a measure of disease severity [13]. Protection from tachypnea is observed in cotton rats immunized with one subtype of influenza A virus and subsequently challenged with another subtype, demonstrating a heterosubtypic immune response. This protection persists despite inhibition of the recruited memory response [14]. The studies presented in this report show that protection is mediated by humoral immunity since passive transfer of immune serum from H1N1-immune animals is able to transfer components necessary for protection from H3N2-induced tachypnea. Protection correlates with HAI titer. While the HAI titer is a measure of a subtype-specific antibodies, it also reflects the total amount of antibody successfully administered during the passive transfer and is therefore likely to correlate with the amount of cross-reactive antibodies present in the serum. These antibodies are most likely specific for conserved epitopes of influenza A, and may include antibodies with specificity for NP, M2e or conserved HA peptides. Non-neutralizing HA-specific antibodies that may contribute to B cell-dependent, heterosubtypic protection against lethal infection by avian H5N1 influenza have been measured in the convalescent sera of mice [9]. While there is good evidence that M2-specific antibodies are induced following infection [17], we were unable to measure anti-M2 titers in our cotton rat serum samples in an ELISA using M2e peptide to coat the plates. The poor sensitivity of this type of assay has been reported and it is known that functional M2e-specific antibodies are best detected using a cell-based expression system [17]. While we do not know the fine specificities of antibodies present in convalescent cotton rat sera, our results show that M2e-specific but not NP-specific monoclonal antibodies can contribute to protection from influenza virus-induced tachypnea.

Further studies are needed to evaluate how antibodies contribute to cross-protection. They may reduce the amount of virus that can attach to cells by directing FcR-positive macrophages to the pathogen for uptake and degradation. A role for macrophages in heterosubtypic immunity is supported by the studies of Sambhara et al. [18]. Alternatively, cross-protective antibodies may work in conjunction with NK cells as demonstrated for protection of mice by M2-specific antibodies [19]. Our finding of antibody-mediated cross-protection against tachypnea in the cotton rat model is an important step toward recognition that this type of response is not limited to mice, and is therefore likely to be present in other animal species, including man.

Our results show that heterosubtypic immunity can be induced by vaccination with either live or inactivated virus that is administered intramuscularly. These results differ from those reported by Tumpey et al. [8] and Takada et al. [20] that show heterosubtypic protection in mice following vaccination with intranasal but not intramuscular-delivery of an inactivated virus vaccine. This latter failure to protect against challenge in mice is likely to reflect the relatively weak responses induced following parental immunization. In our studies three intra-muscular administrations of inactivated virus resulted in HAI titers similar to those obtained following infection; this vaccination regimen was sufficient for heterosubtypic protection supporting the idea that a mucosal IgA response is not necessary for this protection.

Increased respiratory rate is a single facet of influenza disease, and while an antibody-mediated mechanism protects against virus-induced tachypnea in cotton rats, it is likely that other immune mechanisms contribute to protection against other signs of disease. This may include cytokines that have antiviral activity or activate macrophages, and cytotoxic T lymphocytes that play a role in eradicating infected cells. Influenza vaccines that induce a broad range of mechanisms are likely to offer the most effective protection against all influenza A viruses, an important consideration in the development of vaccines designed to induce immunity against highly virulent H5N1 strains with potential for pandemic spread. Our results support the idea that antibodies specific for conserved epitopes play a role in protection from influenza induced disease and are therefore likely to contribute to vaccine efficacy, particularly when HA and NA components are poorly matched with circulating influenza A viruses.

Conclusion

Passive transfer of serum from H1N1-immune cotton rats provides protection against H3N2-induced tachypnea even though the antiserum lacked subtype cross-reactivity in standard HAI, NI or neutralization assays. Since recent studies demonstrate that antibodies contribute to heterosubtypic immunity in mice, these studies in a second animal model support the idea that this mechanism may provide some immune protection against respiratory disease in humans. Such heterosubtypic protection was observed in animals immunized with either live or inacti-

vated virus preparations delivered intranasally or intramuscularly respectively, demonstrating that current human influenza vaccine strategies are likely to induce some heterosubtypic immunity. While the specificity of antibodies that provide cross-protection is have not been fully characterized, our results demonstrate that monoclonal antibodies to M2e but not NP provide some protection against virus-induced tachypnea. This supports the idea that antibodies to conserved epitopes on the surface of the virion or infected cell contribute to heterosubtypic immunity. It is important to establish that similar responses are induced following human vaccination and contribute to vaccine efficacy. Our future studies will therefore characterize the quality and quantity of antibodies that provide heterosubtypic immunity so that tests can be designed to evaluate these responses following human vaccination.

Materials and methods

Cotton rats

Male and female inbred *Sigmodon hispidus* were obtained from a breeding colony maintained at Virion Systems, Inc., Rockville, MD. Animals were seronegative for adventitious viruses. Prior to infection, they were also seronegative for influenza A as tested by HAI assay. Animals were used at 6–12 weeks of age in protocols that follow federal regulations and were approved by the Institutional Animal Care and Use Committee. Animals were sacrificed by CO_2 asphyxiation for the collection of tissue samples.

Viruses

Influenza A/Wuhan/359/95 (A/Wuhan/95), an H3N2 virus, was grown in MDCK cells at Novavax Inc. (Rockville, MD), resulting in a virus stock solution of 10^8 $TCID_{50}$/ml. Tissue culture-adapted influenza A/PR/8/34 (H1N1) was obtained from ATCC, and was grown in a monolayer of MDCK cells resulting in a viral titer of 10^8 $TCID_{50}$/ml. Virus was stored at -70°C, and thawed immediately prior to use. Aliquots of A/PR/8/34 that were exposed to UV-light did not contain any infectious virus.

Measurement of respiratory rates

Respiratory rates (RR) were measured by unrestrained whole body flow plethysmography (Buxco Electronics Inc., Wilmington, NC) as described previously [13]. After calibration of the 2-chamber apparatus (designed to hold adult rats), one cotton rat was placed in each chamber and airway measurements were continuously recorded over a 5-minute period. The mean respiratory rate over the entire 5-minute period was calculated. Data from each group are presented as mean breaths per minute (+/- standard error) or as the percent protection from tachypnea calculated as: $100 - \{100 \times [(RR_{experimental\ group} - RR_{uninfected})/(RR_{primary\ infection} - RR_{uninfected})]\}$.

Hemagglutination inhibition (HAI) assay

Serum was treated with receptor destroying enzyme (RDE) overnight and then serially diluted in PBS. One volume (25 μl) of each dilution was mixed with 1 volume of A/Wuhan/95 containing 4 hemagglutinating units of virus in a U-bottomed 96-well plate. After 30 min incubation at room temperature, 2 volumes of a 0.5% suspension of chicken red blood cells (CBT Farms, Chestertown, MD) were added, the suspension gently mixed and left to settle at room temperature for 30 min. Agglutination was read and the inverse of the last dilution that inhibited agglutination assigned as the titer.

Neuraminidase inhibition (NI) assay

Two-fold dilutions of serum (50 ul per well) were mixed with an equal volume of virus. The amount of virus added provided a signal 10-fold greater than background. Substrate labeled with fluorochrome, 2,4-methylumbelliferone-N-acetyl neuraminic acid (MU-NANA), was then added (100 μl of a 20 μM solution) as previously described for measurement of NA activity [21]. After 1 hr incubation at room temperature the reaction was stopped by addition of 100 ul 0.1 M glycine, pH 10.7 containing 25% EtOH. Fluorescence (365 excitation, 460 emission, 0.1 sec per well) was read on a Victor 3 (Perkin Elmer). The inverse of the last dilution of virus that resulted in at least 50% reduction of NA activity was recorded as the NI titer.

Virus neutralization assay

Serial dilutions of serum were made in DMEM, starting with a 1/100 dilution. An equal volume (100 μl) of virus (200 $TCID_{50}$/ml) was added and the mixture incubated at room temperature for 15 minutes. A portion (100 μl) of the virus-antibody mixture was transferred to duplicate MDCK cell monolayers in 96 well plates that had been washed 3 times with serum-free medium. After 1 hr incubation at 37°C, an equal volume of DMEM containing 1% bovine serum albumin and TPCK-treated trypsin (5 μg/ml) was added to each well, and the plates were returned to the incubator. On day 3 of incubation, the supernatants were discarded and the monolayers fixed and stained with crystal violet. Neutralization titers were assigned as the inverse of the last dilution that inhibited the viral cytopathic effect in both of the duplicate wells. The neutralization assay was also performed in the presence of complement, with addition of 25 μl of a solution of C1q (5 μg/ml) to each well of the tissue culture plate.

Experimental design

Anesthetized animals were immunized by intranasal (i.n.) administration of 10^7 $TCID_{50}$ virus per 100 grams of animal as previously described [22]. This dose of virus is not lethal to cotton rats and corresponds to approximately 100 μl total volume (a 6 week old animal weighs approx-

imately 100 g). This volume is sufficient to deliver the inoculum into the lower respiratory tract, resulting in virus replication in lungs, trachea and nasal tissue. Groups of animals that were not immunized, or immunized with either A/Wuhan/95 (H3N2) or A/PR/8/34 (H1N1) were challenged with the H3N2 virus four weeks later. Sera for transfer studies were obtained from animals never exposed to influenza (naïve control), or exposed to either H3N2 or H1N1 viruses at 3-week intervals 3 times previously. The serum from individual animals in each group were pooled and transferred (0.5 ml per animal) by intraperitoneal injection 24 hr prior to i.n. challenge with virus. Twelve hr before challenge, retro-orbital bleeds were performed on the recipient animals to obtain sera to measure HAI titers. Respiratory rates were measured by whole body plethysmography.

Statistical Analysis

Mean respiratory rates (RR) were compared between groups by non-parametric Kruskal-Wallis and Mann-Whitney tests. All analyses were performed using SPSS (version 13.0) statistical software. *P*-values of <0.05 were considered statistically significant.

Competing interests

The authors declare that they have no financial competing interests. The opinions or assertions contained in this report are the private views of the authors and are not to be construed as reflecting the views of the Uniformed Services University, U.S. Department of the Army, U.S. Department of the Air Force, the U.S. Department of Defense, or the Food and Drug Administration.

Authors' contributions

TMS and MCE designed and executed experiments, analyzed data, and wrote the manuscript. MGO provided substantial input to study design and manuscript preparations. GAP gave final approval for publication. All authors read and approved the final manuscript.

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CHAPTER 4

DISCUSSION

Influenza A virus infection results in protective immunity by way of a neutralizing antibody response to the immunodominant surface glycoprotein viral hemagglutinin (HA), as well as antibodies to neuraminidase (NA) glycoproteins that are specific for homologous influenza A. Heterosubtypic immune responses that offer protection against disease from different influenza A subtypes have been well studied in animal models. Epidemiologic data suggest some heterosubtypic protection in man, but cross-protective antibodies do not appear to be induced after natural infection, and current vaccine approaches are not likely to efficiently elicit this response. Despite years of research in cross-protective immunity, key mechanisms are still controversial and immune correlates of protection have not been established. Early research focused on cross-protective cytotoxic T lymphocytes (CTL), but it has been difficult to induce this type of response consistently with vaccines. Recent research in mice demonstrates that antibodies can contribute to a heterosubtypic immune response in the absence of cell-mediated immune effectors. The development of methods to better induce a cross-reactive antibody response may lead to more broadly-protective influenza vaccines.

The Cotton Rat Model is an Effective Model to Demonstrate and Measure the Heterosubtypic Immune Response to Influenza

In the first series of experiments published out of this body of work, evidence of a heterosubtypic immune response to influenza A is identified and described in detail in the cotton rat (*Sigmodon hispidus*) model. This animal model was chosen due to several characteristics that were attractive features for the purposes of investigating of the immune components contributing to cross-protection. Cotton rats demonstrate predictable physiologic and pathologic responses to primary influenza infection that correlate well with the severity of disease in a semi-permissive model (Ottolini et al. 2005). These results are in contrast to the murine model, where most studies of heterosubtypic immunity report only mortality as the major endpoint. In addition, cotton rats are quite susceptible to a variety of human influenza strains, which can be used without animal-adaptation and avoids alteration of the virus (Ottolini et al. 2005). The viral kinetics and pulmonary histopathology of cotton rats following infection with influenza virus have been described previously, establishing a model to study immunity to influenza (Ottolini et al. 2005). These studies showed that virus replication peaks within 24 hours in the lung (peak titers proportional to the infecting dose) and ceases by day 3; while replication in nasal tissue can persist for 6 days. Pulmonary pathology of infected animals included early bronchiolar epithelial cell damage, followed by extensive alveolar and interstitial pneumonia, which persisted for nearly 3 weeks. Influenza infection in the cotton rat also results in tachypnea for several days, as well as weight loss and decreased temperature (Eichelberger, Prince, and Ottolini 2004, Ottolini et al. 2005). This response to infection can be easily monitored, and respiratory rates can be measured

with whole body plethysmography to provide an objective quantification of respiratory disease (Eichelberger, Prince, and Ottolini 2004). This semi-permissive animal model develops a degree of viral replication and pulmonary pathology proportional to the infecting dose (Ottolini et al. 2005). The degree of tachypnea, a reliable measure of lower respiratory pathophysiology, has also been shown to correlate with the infectious dose of virus administered, as well as subsequent epithelial damage (Eichelberger, Prince, and Ottolini 2004). These features become advantageous in measuring the quality of protection from influenza disease, where a stepwise titration of the degree or severity of infection is important.

In the studies presented here, a heterosubtypic immune response was generated in the cotton rat model following infection with influenza A virus. These results were obtained by choosing measurable endpoints that correlate well with severity of disease and demonstrate significant disease reduction as a manifestation of the protective immune response. Three of these endpoints utilized were reproducible markers of protection against influenza disease severity including: enhanced viral clearance, reduction in tachypnea following infection, and a reduction in respiratory epithelial damage upon pathologic examination of pulmonary tissues.

Cotton rats were infected with influenza A/PR/8/34 (H1N1) or A/Wuhan/359/95 (H3N2), and then challenged with A/Wuhan/359/95(H3N2) virus 4 weeks later. Viral titers were studied in lung and nasal tissues for up to 7 days after virus challenge in immunized and non-immunized animals. Significantly lower viral titers were observed in rats challenged with a heterosubtypic virus after live virus immunization, when compared to primary infection in non-immune animals (Straight et al. 2006, Tables 1 and 2).

Additional experiments were performed to increase the number of time points, and a portion of these data are included in our published work showing that a greater proportion of animals which mounted a heterosubtypic immune response were clear of virus at 48hrs compared to non-immune animals (Straight et al. 2006, Table 2). Unpublished data show that mean viral titers from the lungs were also significantly less in previously immunized cotton rats challenged with heterosubtypic virus at 24 and 36 hours post-infection compared to non-immune animals (Appendix A). From our results, we conclude that enhanced viral clearance from the lungs and nasal turbinates can be used as an indicator of heterosubtypic immunity in cotton rats.

Cotton rats were evaluated by plethysmography to measure respiratory function parameters (including respiratory rate) following heterosubtypic virus challenge. Plethysmography is the measure of several components of pulmonary function through use of a sensitive whole body chamber. Prior published work validated respiratory rate as one of the most consistent measures of lower respiratory disease, correlating with epithelial damage caused by actively replicating virus (Eichelberger, Prince, and Ottolini 2004). With known reference-responses to infection, including responses demonstrating protective immunity to homologous virus, one is able to compare the quality of disease protection based on severity of objectively measured respiratory rates. After immunizing animals with intranasal delivery of live influenza virus, animals challenged with heterosubtypic virus were observed to have significantly decreased levels of tachypnea compared to non-immune animals undergoing primary infection (Straight et al. 2006, Figure 1). The degree of tachypnea following influenza infection in the cotton rat is a unique physiologic endpoint that correlates well with the severity of disease, and

provides a sensitive indicator of protection from disease useful for the study of the quality of immune responses.

Pathologic responses were also observed after primary and secondary infections. Resulting infiltrates from either immune responses or infection, as well as the resultant epithelial damage were scored and compared as reported previously (Ottolini et al. 2005). Immunized animals challenged with homologous virus had early peribronchiolitis (day 1) as a manifestation of a memory immune response in contrast to primary disease, in which little to no early peribronchiolitis was observed. Cotton rats immunized with a virus differing in subtype to the challenge virus had a robust early peribronchiolitis, suggesting a cellular recall response similar to that seen in animals previously immunized with homologous virus. In addition, significantly decreased epithelial damage was noted in the lungs of cotton rats challenged with heterosubtypic virus. Decreased epithelial damage in this setting is likely a consequence of enhanced viral clearance (Straight et al. 2006, Table 3 and Figures 3 and 4). Our data, therefore, show both the early inflammatory response as well as reduced epithelial damage are end-points that can be used to evaluate heterosubtypic immunity in the cotton rat model.

Induction of Heterosubtypic Immunity in the Cotton Rat Model Requires a Substantial Infectious Dose, But is Broadly Protective Among Influenza A Subtypes

Additional experiments were completed to demonstrate the optimal conditions for inducing a heterosubtypic immune response in the cotton rat model, and point toward possible mechanisms involved in the development of such a response. Previous studies have suggested that the quality of heterosubtypic immune response may depend on the

priming dose of virus used to immunize the animal prior to heterosubtypic virus challenge. Variation of immunizing dose and subsequent effect on endpoints (respiratory rate, lung pathology) was evaluated in a study not included in our two publications. This study revealed that the smaller dose of 10^3 $TCID_{50}$ per 100g animal for live virus immunization (delivered intranasally), compared to the typical dose of 10^7 $TCID_{50}$ per 100g animal, failed to protect against virus challenge when compared to primary disease in influenza A/Wuhan-infected cotton rats (Appendix B).

Also, various combinations of priming versus challenge viruses were used to examine the effect on the quality of response. We found no significant difference in protection when exchanging the priming and challenge virus in our experimental protocol, however, there was a trend toward superior heterosubtypic protection from priming with PR8 virus prior to Wuhan challenge (61-63% protection) compared to priming with Wuhan virus prior to PR8 challenge (37-51% protection) as measured by protection from tachypnea (Appendix C).

A variety of influenza A strains were studied as the challenge virus to demonstrate heterosubtypic protection across a broad range of strains within the same heterosubtypic subtype. Protection from disease was established with a variety of influenza A viruses (Straight et al. 2006, Fig 2), but not heterologous influenza B.

Antibody Contributes Significantly to Heterosubtypic Immunity in the Cotton Rat Model and Provides Measurable Protection from Influenza Disease

Using the same endpoints identified in our early studies, additional experiments were performed to assist in the identification of possible components contributing to

heterosubtypic immunity. Previous work had demonstrated that the inflammatory response to influenza infection is significantly reduced with administration of systemic corticosteroids prior to infection, but the influenza-induced tachypnea following infection remains unchanged (Ottolini et al. 2003; Eichelberger, Prince, and Ottolini 2004). Thus, immune and non-immune cotton rats were injected with triamcinolone acetonide and then challenged with influenza A/PR8 (H1N1). Previously immunized animals challenged with heterosubtypic virus retained a significant protection from influenza-induced tachypnea, despite the presence of systemic corticosteroids and blunting of the recruited cellular response as measured by histopathologic scoring (Straight et al. 2006, Fig. 5). These data suggest that immune system components aside from the recruited cellular memory response can contribute to a cross-protective immune response. The possible mechanisms to explain protection in this setting include local or systemic antibodies, since these are not influenced by the use of steroid. Local IgA antibodies at the mucosal surface plays a large role in inhibiting virus replication in the upper respiratory tract (Freihorst and Ogra 2001). In addition, cytokines with anti-viral activity secreted by cells in the lung wound not be impacted by steroid administration, and may therefore, also contribute to heterosubtypic immunity. These cytokines may include interferons (Types I, II, and III) and TNF-α secreted by epithelial cells, tissue macrophages, NK cells, or T cells residing in pulmonary tissues.

The most compelling data in the studies presented here that suggest antibody contributes to the heterosubtypic immune response is the successful passive transfer of immune sera resulting in protection from a heterosubtypic virus challenge *in vivo*. We demonstrated that a reduction in tachypnea after infection with homologous virus or

heterosubtypic virus can be achieved by intra-peritoneal administration of sera from previously infected cotton rats. These experiments also showed that protection (as measured by reduction in tachypnea) correlated with higher titers of antibody. Subsequent experiments were able to show that a minimum titer of 40 against the priming virus resulted in protection from homologous and heterosubtypic virus. This direct correlation between titer of antibody transferred and subsequent quality of protection, although circumstantial, supports the concept of antibody contribution to this type of response (Straight et al. 2008, Figures 1 and 2).

Immunization of animals with inactivated and live virus by way of the intramuscular route achieved similar levels of protection compared to previous intranasal infection with live virus, while intranasal administration of immune sera offered no benefit. Although mucosal antibody may play a role in heterosubtypic immunity, these data suggest that heterosubtypic immunity does not require a mucosal antibody response to protect from disease. These data also support that a cross-reactive cell-mediated response is not necessary for heterosubtypic protection observed under these conditions, since this protection was induced by an inactivated virus preparation (Straight et al. 2008, Figure 4).

Antibodies Contributing to Heterosubtypic Immunity in Cotton Rats Do Not Inhibit Virus Binding or Replication, but Prophylactic Transfer of Monoclonal Antibody to M2e Protects from Heterosubtypic Virus Challenge

Various assays were performed to assess the degree of antibody response and to shed light on the specificity and/or possible function of this cross-reactive antibody

contributing to protection from heterosubtypic virus challenge. Enzyme-linked immunoassays (ELISA) were performed using plates coated with homologous and heterologous influenza viruses. Bronchoalveolar lavage (BAL) and serum samples from cotton rats immunized 28 days previously with influenza A/PR/8/34 or influenza A/Wuhan/95, or naïve cotton rats (negative controls) were evaluated. The antibody titer (defined as the inverse of the dilution of sample that gives an optical density (OD) > 2-fold the OD of the negative control) was determined to be 5120 for both homologous and heterosubtypic sera. BAL samples did not contain sufficient quantities of protein for analysis. A serum titer against heterosubtypic virus indicates that antibodies are present that bind conserved viral gene products (e.g., NP, M) or that have antigenic epitopes that may have cross-reactivity sites (e.g., M2, or perhaps even HA or NA).

These ELISA assays identified antibodies that were able to react with antigens of both the immunizing and challenge virus, and since these are likely specific for abundant conserved viral proteins (nucleoprotein (NP) and matrix (M) protein). However, these results did not provide any information regarding the specificity of the antibodies that are effective *in vivo* or how these antibodies function to protect animals from tachypnea. Therefore, we used additional assays to determine whether cross-reactive antibodies could impede the function of specific viral proteins.

Antibodies specific to HA block binding of virus to the sialic acid-containing receptors on the red blood cell (RBC). Antibodies that are specific for other virus surface proteins may be large enough to interfere with this binding as well; however, antibodies to internal conserved proteins should not interfere with hemagglutination. Hemagglutinin Inhibition Assays (HAI) were performed according to the World Health Organization

protocol using CRBC (chicken red blood cells) to establish antibody titers against influenza A viruses A/Wuhan/359/95 (H3N2) and A/PR/8/34 (H1N1) as described in Straight et al. 2008. Two-fold dilutions were made of each sample, and BAL and sera from animals that had not been infected were used as negative controls. There was no established titer in BAL samples, even to homologous virus, due to poor recovery of antibodies in the samples. Attempts to concentrate BAL samples still failed to produce measurable protein, which was thought to be a consequence of inefficient methods to extract protein from alveolar spaces during the BAL procedure (a common problem in this small animal model). HAI studies of concentrated immune sera used in our published work showed that subtype-specific antibodies were present in sera, as we expected (Straight et al. 2008, Table 1).

Additional techniques were used to evaluate any inhibition of virus binding to the cell surface. A second assay was utilized to attempt to quantify the amount of virus binding taking place. This assay was developed using fluorescently-tagged virus particles. Influenza A viruses were labeled with DiD (1,1'- dioctadecyl-3,3,3',3'tetramethylindodicarbocyanine), a fluorescent lipophilic dye that spontaneously partitions into the viral membrane. The amount of virus bound to cotton rat spleen cell mixture was quantified as mean fluorescence intensity determined by fluorescence microscopy. We evaluated serum samples obtained from cotton rats immunized with either H1N1 influenza A/PR/8/34 or H3N2 influenza A/Wuhan/359/95 (day 28 post-infection), and examined the binding of labeled influenza virus to cotton rat spleen cells in the presence of either immune sera relative to cell mixtures with non-immune sera (Appendix D). The binding assay demonstrated that homologous sera was able to block

50% of virus binding relative to non-immune sera at a dilution of 1:640. There was no significant difference in the amount of binding of virus to the cells in the presence of heterosubtypic-immune sera compared to control samples (containing non-immune sera). These data suggest that it is unlikely that sera from immunized animals contain antibodies that have the ability to block the binding of heterosubtypic virus to cells. However, since this assay examined only the effect of virus binding, it does not shed light on whether antibodies are present that affect virus entry or replication. Such antibodies may act by blocking virus entry (as may be the case for antibodies specific to the conserved fusion peptide of HA), by blocking virus uncoating (as may be the case for antibodies with specificity to M2), by interfering with viral replication (as may be the case for antibodies to NP), or by affecting virus traffic to the epithelium or virus shedding (as may be the case for antibodies to NA).

Since antibodies may act intracellularly to block virus replication, the ability of serum collected from immune cotton rats was evaluated to examine the effect on replication of heterosubtypic viruses. Serum samples from previously infected animals and naïve animals (negative controls) were incubated with homologous and heterologous viruses. The virus mixtures were then applied to Madin-Darby canine kidney (MDCK) cell cultures as part of a neutralization assay. The endpoint ($TCID_{50}$) obtained in the presence of test samples was compared to control. No inhibition of replication of heterosubtypic virus was observed. The assays were repeated with complement component C1q to enhance neutralization of virus. Although the titers for homologous virus increased from 200 to 800 for Wuhan (H3N2), and from 1600 to 3200 for PR8

(H1N1), there was still no measurable inhibition of heterosubtypic virus replication (Straight et al. 2008, Table 1).

Assays to measure neuraminidase activity also revealed that antibodies developed against NA are subtype-specific. The NA inhibition (NI) titer of PR8 (H1N1)-immune sera that had been used in transfer studies was 80 against PR8 (H1N1), and no detectable inhibition was appreciated against the N2 of Wuhan (H3N2). Similarly, the NI titer of Wuhan (H3N2)-immune sera was 320 against homologous virus, and no detectable inhibition against the PR8 (H1N1) virus. It is possible that the serum samples used did not contain certain *in vivo* factors allowing the inhibition of virus binding or inhibition of replication *in vitro* (e.g. complement) or did not contain cells that act through antibody-dependent mechanisms to kill infected cells.

To support the concept that this protection from heterosubtypic immunity may be mediated by antibody to a conserved viral protein, a commercially available monoclonal antibody to the extracellular portion of M2 (M2e) and monoclonal antibody to influenza nucleoprotein (both the likely targets of a cross-protective immune response) were used to determine if either could contribute to this type of immune protection in cotton rats. Subsequent experiments published in Straight et al. 2008 showed that passive transfer of antibody to viral NP did not result in any appreciable level of protection as measured by respiratory rate response to heterosubtypic virus challenge. These data are consistent with previously published studies in mice evaluating antibodies against NP that failed to show protective effect with either passive transfer of antibodies against NP, or utilizing measures to induce anti-NP antibodies (Epstein et al. 1993, Epstein et al. 1997). However, cotton rats administered antibodies specific for viral M2 protein demonstrated

protection from heterosubtypic challenge. These animals had significantly less tachypnea than other animals undergoing primary infection (Straight et al. 2008, Figure 3).

Descriptions of Potentially Broadly Protective Vaccine Targets That May Be Tested in the Cotton Rat Model of Heterosubtypic Immunity

The cotton rat model allows for identification of potentially broadly protective vaccine targets through investigation of their role in heterosubtypic immunity. Our work has demonstrated the protection provided against disease from heterosubtypic influenza A virus with prophylactic administration of anti-M2e antibody. Thus, M2e and other promising antigen targets may be tested for their vaccine potential with this effective animal model.

M2 protein

Although passive transfer of anti-M2 antibody was able to mimic the degree of heterosubtypic protection established in our research using natural infection to immunize animals against influenza, it is unclear whether the transfer of monoclonal anti-M2 antibody represents the same physiologic process *in vivo*. It is likely that the doses of anti-M2 antibody and resultant titer established in to cotton rats for the purposes of the study exceeded that which would be expected to follow natural infection with influenza A. It is clear that passive transfer of anti-NP (another highly conserved antigen target of influenza) did not confer protection from influenza virus in our studies, and anti-HA and anti-NA showed only subtype-specific binding and activity as discussed earlier and demonstrated in Straight et al. 2008. Thus, anti-M2 antibody is an attractive candidate to explain the impressive protection against heterosubtypic virus challenge in our studies of

this immune response in cotton rats. A heterosubtypic immune response mediated by anti-M2 antibody is additionally supported by the transferable nature of this protection from immune animals to non-immune animals by the passive transfer of sera. Although protection against virus challenge correlated with anti-HA antibody measured by host sera HAI as described earlier – this antibody response is subtype specific. The degree of the HAI titer does function as a marker, however, for the presence and concentration total antibody delivered via passive transfer of sera from immune to non-immune animals. In summary, these data support the concept that antibody specific to M2 may be an active component in the immune sera providing protection from heterosubtypic virus challenge in otherwise naïve cotton rats.

Background on M2 protein

Unlike HA and NA, which are constantly changing through antigenic drift, viral M2 protein is a relatively conserved transmembrane protein. M2 protein is a 97 amino-acid long non-glycosylated transmembrane protein forming homotetramers, expressed at low density in the membrane of virus particles: ~10 M2 tetramers compared to ~400 hemagglutinin (HA) trimers and ~100 neuraminidase (NA) tetramers per average virion (Mozdzanowska et al. 2003). Based on the work of Zebedee et al., it is estimated that there are 7 to 85-fold more HA molecules on the surface of influenza A virus than M2 (Zebedee and Lamb1988). However, M2 is well represented on the surface of infected cells approaching the density of HA trimers (Zebedee 1988, Mozdzanowska et al. 2003, Feng et al. 2006).

M2 contains a 24 aa-long, non-glycosylated, N-terminal ectodomain (M2e), while residues 25–43 constitute the transmembrane segment, and the remaining 54 residues

form the cytoplasmic tail (Feng et al. 2006, Fan et al. 2004). The tetrameric M2 protein forms a proton-selective ion channel, which regulates the pH of the viral core during virus entry into the host cell and of transport vesicles that deliver viral transmembrane proteins to the plasma membrane for virus assembly (Fan et al. 2004, Feng et al. 2006).

Antibody to M2e restricts replication and reduces severity of disease in mice

Zebedee and Lamb were first to demonstrate that a monoclonal antibody to the extracellular portion of the M2 protein (M2e) could inhibit growth of the influenza A/Udorn (H3N2) virus as manifested by a reduction in plaque size, however, no such effect on influenza A/WSN (H1N1) virus was observed (Zebedee and Lamb 1988). Treanor et al. further demonstrated that the same monoclonal antibodies to the M2 protein reduced virus replication in the lungs of infected mice as well, showing virus titers were reduced by a factor of 100 following passive transfer of monoclonal M2 antibody into mice prior to challenge with influenza A/Udorn (Treanor et al. 1990, Hughey et al. 1995).

However, expression of the viral M2 gene to induce anti-M2 antibody has not always proven effective. Jakeman et al. used vaccinia-influenza recombinants expressing the M2 gene from A/Udorn/72 (H3N2) to immunize ferrets and found these constructs were not protective upon challenge with homologous virus (Jakeman, Smith, and Sweet 1989). However, the authors were unable to demonstrate the presence of anti-M2 antibodies in sera of immunized animals. Slepushkin et al. later investigated the potential of this conserved transmembrane protein expressed by a baculovirus recombinant (M2 protein of influenza A/Ann Arbor/6/60) in an attempt to induce protective immunity in mice. Vaccination of mice with M2-expressing recombinant resulted in a shorter duration

of virus shedding, and protected mice from a lethal infection with homologous (H2N2) influenza A virus (Slepushkin et al. 1995). Western blot analysis detected antibodies reacting with the M2 protein of purified influenza A/Ann Arbor/6/60 (H3N2) in all immunized mice. In this study, the authors found an antigenic determinant located on the external N-terminus of the M2 protein (M2e), and also observed that vaccination with M2 protected mice from death following a lethal challenge with a heterosubtypic (H3N2) influenza A virus (Slepushkin et al. 1995).

Functional aspects of anti-M2 antibody

Hughey et al. studied the effects of antibody to influenza A virus M2 protein on M2 surface expression and virus assembly (Hughey et al. 1995). The authors proposed the following mechanisms of action for anti-M2 antibody: aggregation of progeny virions at the cell surface in a manner similar to anti-NA antibodies; possible inhibition of ion channel activity, preventing entry into cells and interfering with virus replication under conditions of multiple cycle infection; or finally, anti-M2 antibodies could have a direct affect on virus assembly by interfering with M2 proteins on the surface of infected cells (Zebedee and Lamb 1988, Hughey et al. 1995). These authors found that virus assembly is indeed reduced in a single cycle of infection in the presence of M2 antibody, as well as reduced cell surface expression of M2 protein (Hughey et al. 1995).

Jegerlehner et al. demonstrated that vaccination with a fusion protein of M2 with Hepatitis B core antigen led to protection from influenza challenge that is antibody-mediated, and suggested that antibodies bind to M2 protein expressed on infected cells rather than binding to free virus or neutralizing virus replication (Jegerlehner et al. 2004). These authors reported that the presence of NK cells is important for protection , whereas

complement is not, which suggests that an antibody-dependent cell-mediated cytotoxicity is the likely mechanism of protection (Jegerlehner et al. 2004). These data would be consistent with previous data showing failure of β2M (-/-) mice to be fully protected with use of recombinants expressing M2, as NK cells do not perform efficiently in setting of β2M deficiency (Epstein et al. 1993, Zimmer et al. 1998). The view that antibody-dependent cell-mediated cytotoxicity is a primary mechanism for functional monoclonal M2 antibody would also be consistent with our data presented above, which showed protection *in vivo* with anti-M2 antibody in cotton rats, despite any evidence of virus neutralization with anti-M2 antibody on neutralization assays and the absence of inhibition of virus-to-cell binding in our binding assay.

HA protein

It is possible that antibodies transferred in our studies of immune sera that provided protection from heterosubtypic challenge were specific for a conserved area of HA protein. While we demonstrated only HA-specific binding on HAI assays discussed earlier and published in Straight et al. 2008, antibodies to conserved areas of HA would not result in inhibition of binding on these assays. In addition, our binding assay with labeled virus would likely not appreciate any inhibition of fusion in the setting of antibody to the conserved area of HA either. However, we would have expected to see some neutralizing activity in viral titer data, which we did not observe.

Background on HA2

Influenza A virus hemagglutinin (HA) has two structurally distinct regions: the antigenically variable globular head, which contains a receptor binding site that is responsible for virus attachment to the target cell (HA1); and the highly conserved stem

(HA2), which contains a fusion peptide that induces membrane fusion between the virus and the cell (Sagawa et al. 1996). Since the specifically targeted epitopes of the stem region of HA play a critical role in membrane fusion, allowing for virus entry into cells, their conserved amino acid sequence is expected (Ekiert et al. 2009). This functional requirement also means these epitopes are less likely affected in the setting of a new circulating antigenic drift or shift variant. Heterosubtypic activity of antibodies to a conserved region of HA were described first by Okuno et al. in 1993, which described the presence of conserved antigenic sites on HA in two different subtypes of influenza A virus (Okuno et al. 1993). Authors noted that antibody directed at this region had neutralizing activity against all of the H1 and H2 strains by inhibition of fusion, but did not show hemagglutination inhibition activity. Since that time, antibodies with specificity for a conserved region of HA have been shown to provide relatively broad protective immunity to influenza A challenge in a number of studies (Sagawa et al. 1996, Wei et al. 2010, Steel et al. 2010). Antibody against HA2 likely neutralizes the virus by blocking conformational rearrangements associated with membrane fusion (Ekiert et al. 2009).

Future Implications of This Work and Its Applications in Other Research

With recent publicized threats of newly emerging influenza A strains, and the limitations of the seasonal vaccine to provide broad protection against these new viruses, there is an even greater push for a universal vaccine that will induce protection against a wide variety of influenza A subtypes. The cotton rat is a valuable animal model in the study of host immune response to influenza A virus, and we have demonstrated a

heterosubtypic immune response to influenza A virus challenge. The mechanism of the heterosubtypic immune response can be further explored in the cotton rat model. A variety of expression systems and adjuvants can be quickly tested to provide candidate vaccines that induce a broadly active immune response with heterosubtypic protection from disease. The ability to avert or reduce the impact of disease from novel strains of influenza A virus wound be a great benefit to public health.

There are several reasons in the literature why viral M2 protein is a tantalizing choice for a universal vaccine target: antibodies directed against its extracellular domain (M2e) have been shown to restrict virus replication and reduce severity of disease in animal models, M2e also shows remarkable conservation amongst human influenza A strains, and lastly, humans appear to lack M2e-specific antibody-mediated protection (Feng et al. 2006). Several studies of M2 protein have outlined the high degree of conservation among different subtypes of influenza A. Feng et al. compared the M2 protein amino acid composition of 1505 influenza A virus strains isolated from humans between 1918 and 2005, and found very little diversity among human isolates including H1N1, H1N2, H3N2, H5N1, and H9N2 subtypes (Feng et al. 2006). In particular, the extracellular portion of M2 (M2e, 23 amino acids long) is highly conserved in its 9 amino acid N-terminal end with the majority of human isolates actually sharing the same sequence (Mozdzanowska et al. 2003). Liu et al. compared the amino acid sequences of M2e protein, and found that among the 188 strains of influenza A with available M2e sequences at the time, there were no differences among the first 9 amino acids (Liu, Li, and Chen 2003).

It is not surprising, given the annual epidemics of influenza, that natural infection in humans does not appear to generate a significant antibody response to M2 protein. Although M2-specific antibody can be detected in serum from individuals recently infected with influenza A virus, the response is apparently transient and occurs only in some adults infected with influenza (Black et al. 1993, Frace et al. 1999). Black et al. were able to identify anti-M2 antibody present in the sera of 12 out of a total of 17 patients infected with influenza A virus with a Western blot assay using the baculovirus-expressed M2 protein (Black et al. 1993). Liu et al. studied whether antibodies to the extracellular portion of M2 were present in higher quantity in patients following influenza infection than in those patients with a "negative" HAI titer against influenza (Liu, Li, and Chen 2003). These authors found no significant difference in the presence of M2e-specific antibody between these two groups. Feng et al. in 2006 discussed two previous studies by Johansson et al. using ELISA and Western blot against M2e peptides and recombinant M2 protein as immunosorbents that reported titers to be low or undetectable, but pointed out that these assays may not have detected all antibodies capable of binding to native tetrameric M2e (Feng et al. 2006, Johansson et al. 1987). The Western blot used by Johansson et al. in a study of 17 paired serum samples from acute and convalescent cases of human influenza appeared to be more sensitive than ELISA, as it detected M2-specific Abs in 13 (70%) convalescent serum samples versus only 5 cases detected with ELISA as defined by a >2 fold increase in titer (Johansson et al. 1987). In a later study using ELISA against M2e peptide, no significant differences were noted between 66 patients with influenza and 44 influenza "negative" individuals (Johansson and Kilbourne 1993). Feng et al. used a cell-based ELISA to quantify

antibodies that could bind to native conformation of M2e, and found that in patients presenting with naturally acquired influenza virus infection, only 11 of 24 paired sera showed a ≥ 4-fold increase in M2e-specific antibody titer (Feng et al. 2006). The authors concluded that M2e-specific antibody-mediated protection is currently lacking or suboptimal in humans (Feng et al. 2006). Natural infection does not appear to generate a significant antibody response to M2 protein based on these data. There is evidence that M2e is also targeted by the cell mediated immune response, as it is expressed on the surface of virus-infected cells, but neither current subunit nor inactivated whole virus vaccines induce this response to a significant degree (Mozdzanowska et al. 2003; Jameson, Cruz, and Ennis 1998; Gianfrani et al. 2000).

Similarly, neutralizing antibodies that recognize the stem region of HA (HA2) are broadly cross-protective across strains and subtypes of influenza A, but are not induced by virus infections or by current influenza vaccines (Wiley, Wilson, and Skehel 1981). Antibodies to the conserved epitope HA2 are not likely to be produced by natural infection given their position relative to the large globular head of HA1, which dominates the immune response (Steel et al. 2010). Removal of this globular head of HA1 and subsequent immunization with “headless HA” prior to challenge with lethal influenza virus A/FM/1/47 (HIN1) in mice, resulted in significantly higher survival rates when compared to controls (Sagawa et al. 1996).

Given the absence of cross-protective antibodies to these conserved areas following natural infection or current vaccination strategies, vaccines to induce heterosubtypic immunity will likely require the use of novel formulations, adjuvants, or delivery methods to increase the immunogenicity of the conserved components.

Conjugate vaccines have been formulated with M2 protein combined with a carrier to increase the immune response to the desired antigen. The immunogenicity of M2 has indeed been increased by fusion with glutathione S-transferase fusion protein (Frace et al. 1999), keyhole limpet hemocyanin or *Neisseria meningitidis* outer membrane protein (Fan et al. 2004), or the hepatitis B virus core (HBc) protein (Neirynck et al. 1999). These strategies increased heterosubtypic protection against a lethal virus challenge that was also transferable by serum (Neirynck et al. 1999). Although it is difficult to compare the different studies evaluating M2-targeting efforts with regard to immunogenicity or strength of protection from disease, they establish that induction of M2-specific immunity can provide significant resistance against influenza A in mice (Mozdzanowska et al. 2003). The additional use of an adjuvant like aluminum hydroxide with such conjugate vaccines may further increase immunogenicity and subsequent antibody production by increasing antigen presenting cell uptake, and possibly delaying antigen release providing a longer exposure to the desired immunogen, but would likely not enhance the cell-mediated response to the target antigen (HogenEsch 2002). Various other adjuvants that may enhance the immunogenicity of mucosally-administered antigens (Ogra, Faden, and Welliver 2001) could also be tested in the cotton rat model for their ability to improve the effectiveness of a heterosubtypic vaccine.

DNA vaccines also have been utilized to provide an alternative mechanism of vaccination, which enables host cells to produce the target antigen after plasmid delivery. The expressed target antigen at the host cell surface allows for activation of antigen-specific B cells, as well as antigen presentation to initiate a cell-mediated immune response. Wei et al. used a vaccination with plasmid DNA encoding H1N1 influenza

hemagglutinin (HA) and boosting with seasonal vaccine or replication-defective adenovirus vector encoding HA, and stimulated the production of broadly neutralizing influenza antibodies. This prime/boost combination demonstrated neutralization of a diverse group of previously circulating H1N1 strains, and showed protection against divergent H1N1 viruses in mice and ferrets.

However, not all influenza A viruses can be expected to cross-react with M2e antigen. In a study of M2e-carrier conjugate vaccines, serum antibodies specific for M2e-con or M2e-A/PR/8/34 (H1N1) did not cross-react with M2e peptides from H5 and H7 subtype avian viruses that have 3 or 4 mismatches (Tompkins et al. 2007). Similarly, there are differences in the HA2 sequence that impact binding of HA-specific cross-reactive antibodies. A mixture of M2 and HA peptides or proteins would ensure all influenza strains are targeted by a universal vaccine.

Despite effective vaccine studies showing broad protection from a wide variety of influenza A strains, there are data to show that escape mutants occur in this setting. The high degree of structural conservation of M2e could in part be the consequence of a poor M2e-specific antibody response and thus the absence of pressure for change (Zharikova et al. 2005). In experiments examining the possibility of escape mutants, the course of infection in SCID mice in the presence or absence of passive M2e-specific monoclonal antibodies was studied, and virus mutants with antigenic changes in M2e emerged in 65% of virus-infected mice treated with M2e-specific (but not control) monoclonal antibodies (Zharikova et al. 2005). Zebedee and Lamb noted in 1989 that influenza A virus variants selected for resistance to M2 antibody inhibition exhibited only single point-mutations in the cytoplasmic domain of M2 or at one of two sites in the M1 protein (Zebedee and

Lamb 1989, Hughey et al. 1995). For this reason, universal vaccine programs should still include surveillance for possible escape mutants in order to update vaccines when necessary.

CONCLUSION

Influenza A virus poses a constant threat of pandemic disease through emerging subtypes not previously circulated in man. Current seasonal vaccine strategies require vigilant surveillance, annual updating, and offer little protection in the setting of a novel influenza A virus. If previous pandemic influenza is used as a model, an effective "universal" vaccine to broadly protect against these new and emerging strains could save millions of lives. Although induction of a cross-protective response in humans may be minimal in the setting of natural infection, strategies to develop such broadly-acting protection are thought to be feasible because highly controlled infectious challenges in animal models provide evidence of naturally occurring cross-protective immunity. In these studies, we demonstrated the characteristics of a cross-protective immune response in cotton rats, and determined the contribution of antibody to heterosubtypic immunity in this new animal model. It is possible that either extracellular portions of M2 protein (M2e) or conserved regions of hemagglutinin (HA) play a role in this protection. Because of their poor immunogenicity when used alone, vaccine studies to induce antibodies to conserved regions of HA or M2 should consider the use of conjugate vaccine methods and adjuvants to increase immunogenicity. As with any vaccine, the safety and efficacy of these novel vaccines will need to be tested in an animal model prior to clinical trials. The cotton rat model we have established presents a system to accomplish such testing in addition to identifying immune mechanisms that contribute to protection.

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Appendix A

Additional experiments were performed to increase the number of time points, methods were as described in Straight et al 2006. Data from these experiments are reported as Table 2 from Straight et al 2006 in a format showing that a greater proportion of animals who mounted a heterosubtypic immune response were clear of virus at 48hrs compared to non-immune animals. To further demonstrate the kinetics of viral clearance in these studies, the data presented here shows mean viral titers (+/- SEM) for each group at various time points (5 animals/group at each time point). The figure legend within the graph (Figure 1) represents either the influenza A challenge virus used in naïve animals (Wuhan, H3N2) or the priming/challenge virus used (Wuhan, H3N2; or PR8, H1N1). These studies show that the mean viral titers for H1N1-immune animals challenged with heterosubtypic virus (H3N2) were significantly less than non-immune animals challenged with Wuhan (H3N2) virus at the 24 and 36 hour time points.

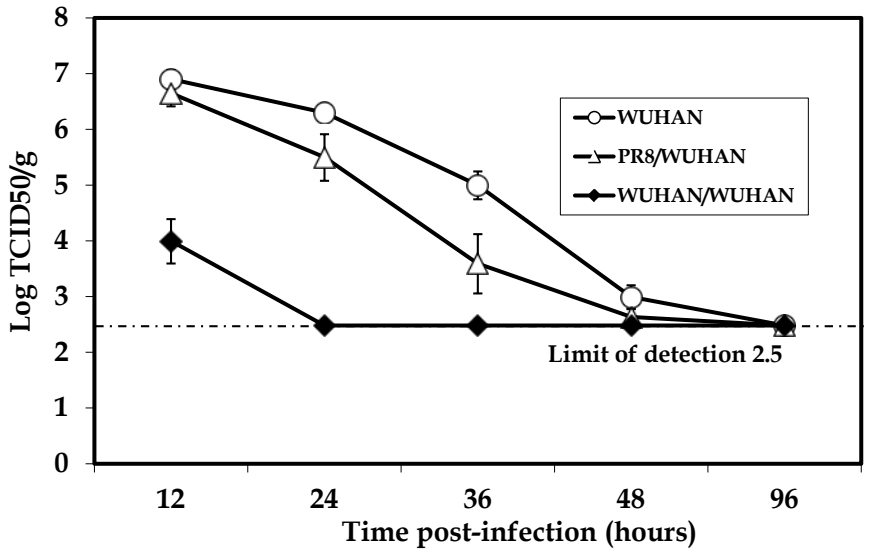
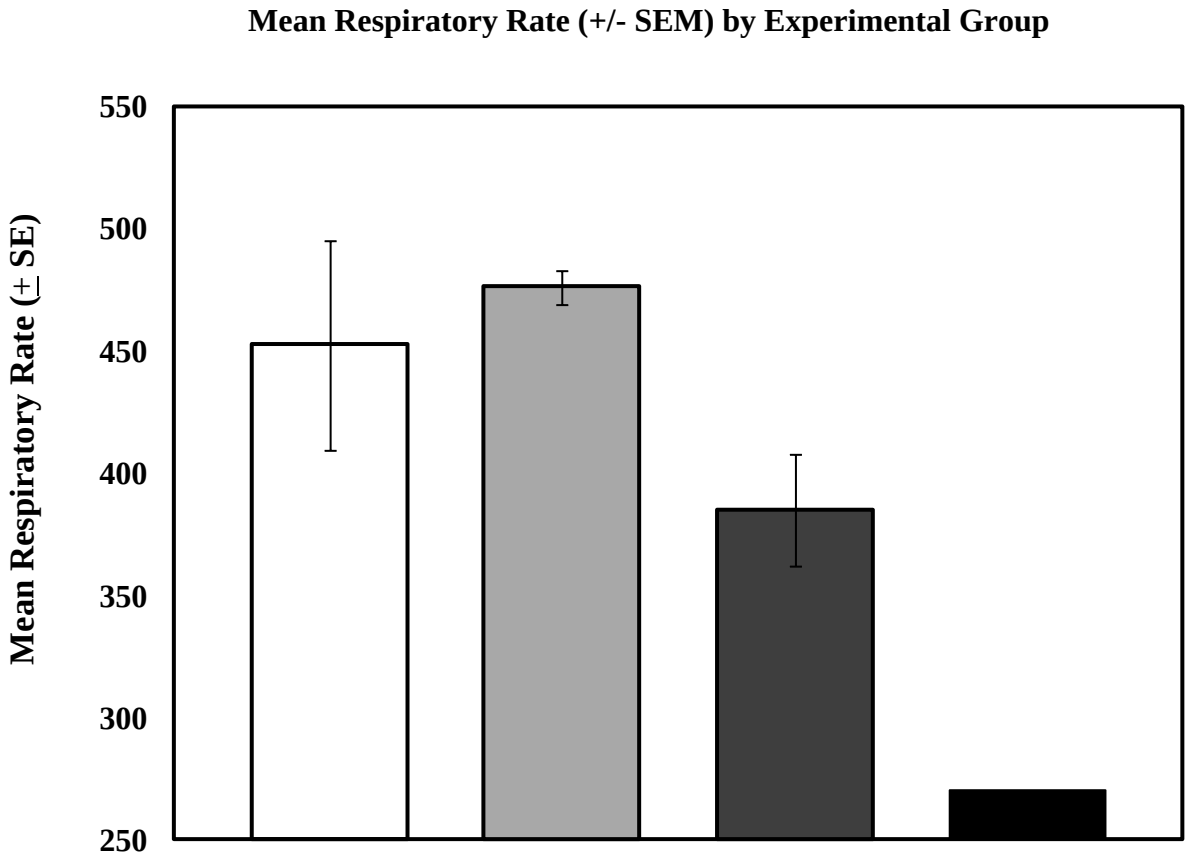


Figure 1. Mean viral titers (±SEM) following either primary infection with A/Wuhan (H3N2) or challenge with A/Wuhan (H3N2) after immunization with either heterosubtypic (A/PR8, H1N1) or homologous (A/Wuhan, H3N2) virus at various time points post-infection. Mean viral titers for H1N1-immune animals challenged with heterosubtypic virus (H3N2) were significantly less than non-immune animals at the 24 and 36 hour time points post-infection.

Appendix B

Variation of immunizing dose and subsequent effect on endpoints (respiratory rate, lung pathology) was evaluated in a study not included in the two published papers. Methods used for the study were similar to those previously reported in Straight et al 2006. This study revealed that the smaller intranasal dose of 10^3 $TCID_{50}$ per 100g animal for live virus immunization, represented by "Wuhan (low dose)" in Figure 2, failed to protect against virus challenge when compared to primary disease in Wuhan-infected cotton rats as measured by respiratory rate.



	n=4	n=4	n=5	n=20
Priming Virus	None	Wuhan (low dose)	Wuhan	None
Challenge Virus	PR8	PR8	PR8	None

Figure 2. Mean respiratory rate (±SEM) following influenza A/PR8 (H1N1) challenge. Respiratory rates measured by whole body plethysmography on day 2 post-infection are shown for groups either not immune or immunized by low-dose or high-dose A/Wuhan (H3N2). Cotton rats immunized with low dose influenza A/Wuhan (H3N2) did not demonstrate protection from tachypnea compared to non-immune rats upon challenge with influenza PR/8 (H1N1) virus.

Appendix C

Multiple experiments were performed evaluating respiratory rate following response to virus challenge as a measure of protection from disease as reported in Straight et al 2006. Methods used in experimental models, and calculations of percent protection, as well as statistical methods were as described in Straight et al 2006. A trend was observed toward superior heterosubtypic protection against tachypnea after priming with influenza A/PR8 (H1N1) virus prior to influenza A/Wuhan (H3N2) challenge (61-63% protection) compared to priming with Wuhan (H3N2) virus prior to PR8 (H1N1) challenge (37-51% protection). Examples of results from multiple animal experiments are shown in Table 1 by study group (represented by priming virus used/challenge virus used) with mean respiratory rates (RR +/- SEM), as well as percent protection from disease, and the p value as compared to non-immune animals undergoing primary infection. Note that all study groups for each experiment are not represented in Table 1.

Table 1. Mean respiratory rates (RR ±SEM) and mean percent protection (%) on day 2 post-challenge in cotton rats immunized (primed) with either A/PR8 or A/Wuhan.

Study Title	**n**	**Mean RR +/- SEM**	**% Protection**	**p***
B 52 (priming/challenge)				
None/Wu	3	451 ± 19	0	
PR8 / Wu	4	339 ± 19	63	p<0.02
Wu/PR8	3	360± 14	51	p<0.02
Wu /Wu	4	361 ± 20	51	p<0.02
Controls	20	273 ± 5		
B 55 (priming/challenge)				
None/PR8	4	452 ± 39	0	
Wu / PR8	5	385 ± 23	37	p=0.07
B 59 (priming/challenge)				
None/PR8	5	511 ± 22	0	
Wu / PR8	5	407 + 35	44	p<0.04
B 61 (priming/challenge)				
None/Wu	5	476 ± 23	0	
PR8 / Wu	5	352 ± 16	61	p<0.01
Wu /Wu	5	297 + 13	88	p<0.01

**compared to respiratory rate of non-immune animals undergoing primary infection*

Appendix D

Influenza A viruses were labeled with 1,1'-dioctadecyl 3,3,3',3'tetramethylindodicarbocyanine (DiD), a fluorescent lipophilic dye that spontaneously partitions into the viral membrane. DiD-labeled virus was added to cotton rat (CR) spleen cells in phosphate buffered saline. The ratio of viral particles to cell started at 1:1 with 10 fold dilutions for titration. Mixtures of virus and cells were then incubated at room temperature for 10 min to allow binding to cells to occur without endocytosis. Unbound label was dialyzed off of the cell mixtures at 4°C with 10,000 MWCU dialysis cups for 4hrs using HEPES buffer (1M) as the dialysate. Samples were assessed by FACS (fluorescence activated cell sorting) to separate cells with bound virus (labeled) and provide a mean fluorescence. An arbitrary threshold for background noise was established, which left only 2% of the cell population beyond threshold in a cell-only mixture, 7% of the cell population in a cell mixture containing unbound label, and 40% with bound and labeled virus as demonstrated in Figure 3. Further experiments evaluated RDE (receptor destroying enzyme)-treated and heat inactivated sera from immunized animals (by either heterosubtypic or homologous virus) and non-immunized animals by adding study samples to known virus titration incubating for 60min prior to applying to spleen cells. Homologous sera demonstrated inhibition of virus binding to cells at even low concentrations of antibody, but results from heterosubtypic-immune sera was similar to that of unimmunized cotton rats (Figure 4A). Each dilution of sample was compared to control to determine a relative binding value. These results showed that Wuhan-immune serum was able to block binding of labeled virus in comparably small quantities (1:640) with 50% binding relative to control dilutions containing non-immune sera. In contrast, PR8-immune serum was not able to significantly block binding of labeled virus to cells in the setting of heterosubtypic virus even at the highest concentration of sera (Figure 4B).

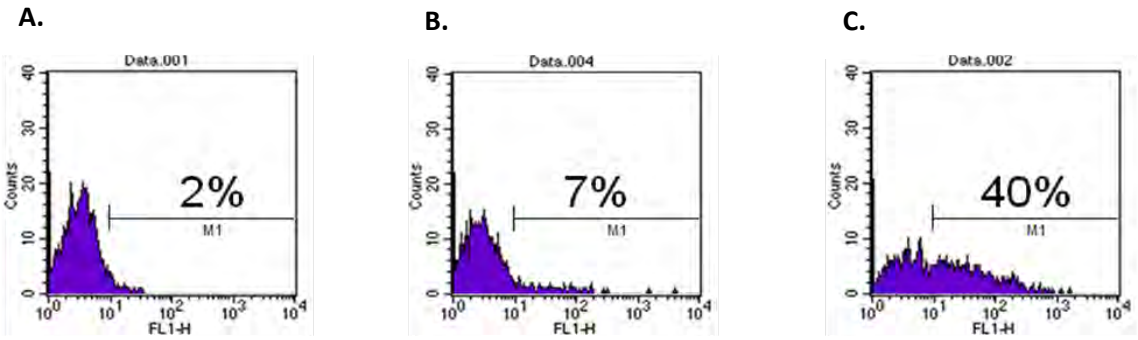
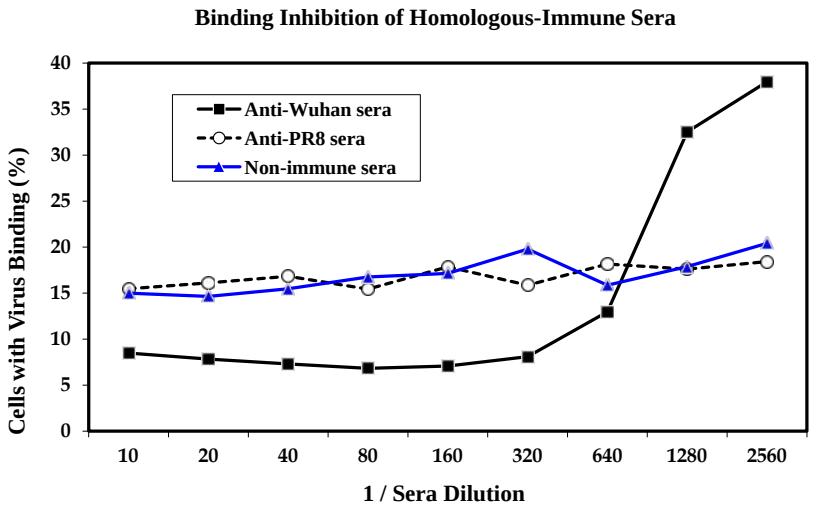


Figure 3. Flow cytometry analysis showing cotton rat spleen cells (CRSp) with either A.) CRSp alone, B.) CRSp with unbound dye, or C.) CRSp with labeled-virus bound to cells. The percentage of cells in the target range for bound, labeled virus is provided within the graph.

A.



B.

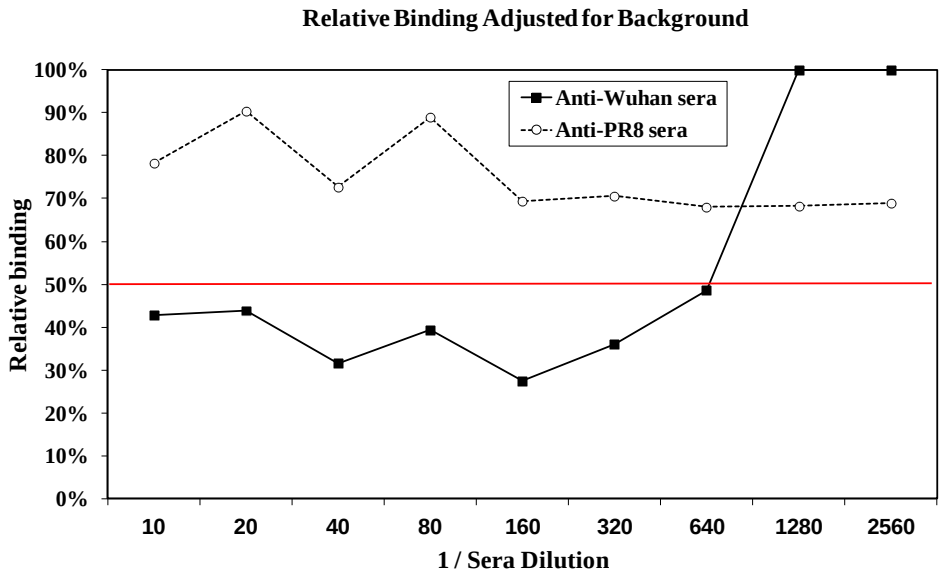


Figure 4. Binding assay demonstrating: A.) effective inhibition of virus binding to cells by homologous-immune sera compared to either heterosubtypic-immune or non-immune sera, and B.) homologous sera (anti-Wuhan sera) reaching a 50% relative binding endpoint at a titer of 640 compared to heterosubtypic-immune sera, which failed to reach the relative binding endpoint.